

5 THE USE OF INHIBITORS OF INDOLEAMINE-2,3-DIOXYGENASE IN COMBINATION
 WITH OTHER THERAPEUTIC MODALITIES

This application claims the benefit of U.S. Provisional Application Serial No. 60/459,489, filed April 1, 2003, and U.S. Provisional Application Serial No. 60/538,647, filed 10 January 22, 2004. Both of these provisional applications are incorporated herein by reference in their entireties.

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BACKGROUND

The adaptive immune system must tailor the T cell repertoire so as not to respond to self-20 antigens. The classical model (reviewed by Nossal in *Cell* 1994;76:229-239) holds that autoreactive T cell clones are deleted in the thymus via a process of negative selection in which encounter with antigen at the immature thymocyte stage triggers apoptosis, resulting in clonal deletion. Although the thymus undoubtedly provides a major site of negative selection, there are difficulties with this model. First, it would seem unlikely that every developing T cell could be 25 exposed to every self-antigen during its relatively brief transit through the thymus. Second, autoreactive T cells are empirically found in the peripheral blood of normal, healthy hosts (Steinman, *Cell* 1995;80:7-10). This suggests the existence of additional means of tailoring the T cell repertoire after the T cells have left the thymus, a process designated as peripheral tolerance.

30 The immune system of a tumor-bearing host often fails to respond protectively against tumor antigens. Functionally, the host is tolerant toward the tumor (Smyth et al., *Nat. Immunol.*,

2001;2:293). This is not due to a peculiarity of tumor antigens, because even highly immunogenic viral proteins become tolerizing when introduced on tumor cells (Staveley-O'Carroll et al., *Proc. Natl. Acad. Sci. USA*, 1998;95:1178). Tumor-induced tolerance is actively created and is maintained in an ongoing fashion (Sotomayor et al., *Blood*, 2001;98:1070; and 5 Cuenca et al., *Cancer Res.*, 2003;63:9007). Thus, tumors represent a striking and biologically significant example of acquired peripheral tolerance (Pardoll, *Ann. Rev. Immunol.*, 2003; 21:807). The molecular mechanisms by which this tolerance arises are currently unclear. This tolerance allows tumors to escape the host's normal immune surveillance and imposes a fundamental barrier to successful clinical immunotherapy.

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SUMMARY OF THE INVENTION

The present invention includes a method of treating a subject with a cancer or an infection, the method including administering to the subject an inhibitor of indoleamine-2,3-dioxygenase in an amount effective to reverse indoleamine-2,3-dioxygenase-mediated 15 immunosuppression, and administering at least one additional therapeutic agent wherein the administration of the inhibitor of indoleamine-2,3-dioxygenase and the at least one additional therapeutic agent demonstrate therapeutic synergy. In some embodiments of the method, the indoleamine-2,3-dioxygenase-mediated immunosuppression is mediated by an antigen-presenting cell (APC).

20 In some embodiments of the method of the present invention, at least one additional therapeutic agent is an antineoplastic chemotherapy agent, including, for example, cyclophosphamide, methotrexate, fluorouracil, doxorubicin, vincristine, ifosfamide, cisplatin, gemcytabine, busulfan, ara-C, or combinations thereof.

25 In some embodiments of the method of the present invention, the additional therapeutic agent is radiation therapy, including, for example, localized radiation therapy delivered to the tumor and total body irradiation.

30 In some embodiments of the method of the present invention, the inhibitor of indoleamine-2,3-dioxygenase may be 1-methyl-tryptophan, β -(3-benzofuranyl)-alanine, β -(3-benzo(b)thienyl)-alanine, or 6-nitro-D-tryptophan. In some embodiments, the inhibitor of indoleamine-2,3-dioxygenase is a D isomer of an inhibitor of indoleamine-2,3-dioxygenase, including, for example, the D isomer of 1-methyl-tryptophan, the D isomer of β -(3-

benzofuranyl)-alanine, the D isomer of β -(3-benzo(b)thienyl)-alanine, or the D isomer of 6-nitro-D-tryptophan.

In some embodiments of the method of the present invention, the cancer is melanoma, colon cancer, pancreatic cancer, breast cancer, prostate cancer, lung cancer, leukemia, brain tumors, lymphoma, sarcoma, ovarian cancer, or Kaposi's sarcoma.

5 In some embodiments of the method of the present invention, the method further includes bone marrow transplantation or peripheral blood stem cell transplantation.

In some embodiments of the method of the present invention, the infection may be a viral infection, infection with an intracellular parasite, or an infection with an intracellular bacteria. In 10 some embodiments, the viral infection is human immunodeficiency virus or cytomegalovirus. In some embodiments, the intracellular parasite may be *Leishmania donovani*, *Leishmania tropica*, *Leishmania major*, *Leishmania aethiopica*, *Leishmania mexicana*, *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, or *Plasmodium malariae*. In some embodiments, the intracellular bacteria may be *Mycobacterium leprae*, *Mycobacterium tuberculosis*, *Listeria monocytogenes*, or *Toxoplasma gondii*.

15 In some embodiments of the method of the present invention, the additional therapeutic agent is a vaccine. In some embodiments the vaccine may be an anti-viral vaccine, including, for example, a vaccine is against HIV. In some embodiments the vaccine is against tuberculosis or malaria. In some embodiments the vaccine is a tumor vaccine, including, for example, a 20 melanoma vaccine. In some embodiments the tumor vaccine includes genetically modified tumor cells or a genetically modified cell line, including genetically modified tumor cells or genetically modified cell line that have been transfected to express granulocyte-macrophage stimulating factor (GM-CSF). In some embodiments the vaccine includes one or more immunogenic peptides. In some embodiments the vaccine includes dendritic cells.

25 In some embodiments of the method of the present invention, the additional therapeutic agent is a cytokine, including, for example granulocyte-macrophage colony stimulating factor (GM-CSF) or flt3-ligand.

30 The present invention also includes a method of augmenting the rejection of tumor cells in a subject, the method including administering an inhibitor of indoleamine-2,3-dioxygenase and administering at least one antineoplastic chemotherapeutic agent, wherein the rejection of tumor cells obtained by administering both the inhibitor of indoleamine-2,3-dioxygenase and the

antineoplastic chemotherapeutic agent is greater than that obtained by administering either the inhibitor of indoleamine-2,3-dioxygenase or the antineoplastic chemotherapeutic agent alone.

The present invention also includes a method of treating cancer, the method including administering an inhibitor of indoleamine-2,3-dioxygenase and administering at least one antineoplastic chemotherapeutic agent, wherein cancer survival rate observed by administering both the inhibitor of indoleamine-2,3-dioxygenase and the antineoplastic chemotherapeutic agent is greater than the cancer survival rate observed by administering either the inhibitor of indoleamine-2,3-dioxygenase or the antineoplastic chemotherapeutic agent alone.

The present invention also includes a method of reducing tumor size or slowing tumor growth, the method including administering an inhibitor of indoleamine-2,3-dioxygenase and administering at least one antineoplastic chemotherapeutic agent, wherein the tumor size or tumor growth observed with the administration of both the inhibitor of indoleamine-2,3-dioxygenase and the antineoplastic chemotherapeutic agent is less than the tumor size or tumor growth observed with the administration of either the inhibitor of indoleamine-2,3-dioxygenase or the antineoplastic chemotherapeutic agent alone.

The present invention also includes a method of augmenting rejection of tumor cells in a subject, the method including administering an inhibitor of indoleamine-2,3-dioxygenase and administering radiation therapy, wherein the rejection of tumor cells wherein the rejection of tumor cells obtained by administering both the inhibitor of indoleamine-2,3-dioxygenase and the radiation therapy is greater than that obtained by administering either the inhibitor of indoleamine-2,3-dioxygenase or the radiation therapy alone.

The present invention also includes a method of treating cancer, the method including administering an inhibitor of indoleamine-2,3-dioxygenase and administering radiation therapy, wherein the cancer survival rate observed by administering both the inhibitor of indoleamine-2,3-dioxygenase and radiation therapy is greater than the cancer survival rate observed by administering either the inhibitor of indoleamine-2,3-dioxygenase or radiation therapy alone.

The present invention also includes a method of reducing tumor size or tumor growth, the method including administering an inhibitor of indoleamine-2,3-dioxygenase and administering radiation therapy, wherein the tumor size or tumor growth observed with the administration of both the inhibitor of indoleamine-2,3-dioxygenase and radiation therapy is less than the tumor

size or tumor growth observed with the administration of either the inhibitor of indoleamine-2 3-dioxygenase or-radiation therapy alone.

The present invention also includes a method of treating an infection, the method including administering an inhibitor of indoleamine-2,3-dioxygenase and administering at least 5 one additional therapeutic agent, wherein a symptom of infection observed after administering both the inhibitor of indoleamine-2,3-dioxygenase and the additional therapeutic agent is improved over the same symptom of infection observed after administering either the inhibitor of indoleamine-2,3-dioxygenase or the additional therapeutic agent alone. IN some embodiments, the additional therapeutic agent is an antiviral agent, an antibiotic, an antimicrobial agent, a 10 cytokine, or a vaccine. In some embodiments, the a symptom of infection observed may be reduction in viral load, increase in CD4⁺ T cell count, decrease in opportunistic infections, increased survival time, eradication of chronic infection, or a combination thereof.

The present invention also includes a method of treating a subject receiving a bone marrow transplant or peripheral blood stem cell transplant by administering an inhibitor of 15 indoleamine-2,3-dioxygenase. In some embodiments of the method, the inhibitor of indoleamine-2,3-dioxygenase is administered in an amount effective to increase the delayed type hypersensitivity reaction to tumor antigen, delay the time to relapse of post-transplant malignancy, increase relapse free survival time post-transplant, and/or increase long-term post-transplant survival. In some embodiments of the method the inhibitor of indoleamine-2,3- 20 dioxygenase is administered prior to full hematopoietic reconstitution.

Definitions

As used herein, the term “subject” represents an organism, including, for example, an animal. An animal includes, but is not limited to, a human, a non-human primate, a horse, a pig, 25 a goat, a cow, a rodent, such as, but not limited to, a rat or a mouse, or a domestic pet, such as, but not limited to, a dog or a cat.

As used herein “*in vitro*” is in cell culture, “*ex vivo*” is a cell that has been removed from the body of a subject, and “*in vivo*” is within the body of a subject.

As used herein, “treatment” or “treating” include both therapeutic and prophylactic 30 treatments.

Unless otherwise specified, “a,” “an,” “the,” and “at least one” are used interchangeably and mean one or more than one.

BRIEF DESCRIPTION OF THE FIGURES

5 Figures 1A-1D. Expression of indoleamine 2,3-dioxygenase (IDO) by antigen-presenting cells (APC). In Fig. 1A human monocytes were analyzed without culture (fresh, $n = 12$); cultured for 7 days in MCSF with IFN- γ added for the final 18 hours (M ϕ + IFN γ , $n = 8$); or cultured in granulocyte-macrophage CSF + IL-4 (DCs) in BCS medium ($n = 34$) or SFM ($n = 24$). Upper row represents IDO versus CD123; lower row represents CCR6 versus CD123 on the same triple-stained cells. Negative control for IDO staining was the primary antibody preadsorbed with the immunizing peptide. Fig. 1B represents immunophenotype of nonadherent (IDO $^+$) dendritic cells (DCs) (dark lines) versus adherent cells (light lines) from SFM cultures, matured with tumor necrosis factor- α /IL-1 β /IL-6/prostaglandin E₂. Fig. 1C represents morphology of adherent (left) and nonadherent (right) cells (cytocentrifuge preparations, 10 Wright’s stain; scale bar, 10 μ m). Fig. 1D represents immunophenotype of MCSF-derived M ϕ s, 15 gated separately on the CD123 (dark lines) and CD123NEG (light lines) populations.

Figures 2A-2I. Effect of DC maturation on IDO expression. In Fig. 2A monocyte-derived M ϕ s were analyzed with (left) or without (right) IFN- γ added for the final 18 hours. Fig. 2B represents DCs (BCS system), with or without IFN- γ for 18 hours. Fig. 2C represents 20 DCs matured with antibody to CD40 on days 5 to 7, with or without IFN- γ for 18 hours. Fig. 2D represents DCs matured with antibody to CD40 on days 5 to 7, with or without IFN- γ for 18 hours, and with IL-10 added during the maturation step. Fig. 2E presents functional enzymatic activity. Depletion of tryptophan from the culture medium (expressed as a percentage of the starting tryptophan concentration in fresh medium, 25 μ M) by DCs with or without IFN- γ for 18 25 hours. Immature DCs (iDC), CD40-matured DCs (mDC), and CD40-matured DCs in the presence of IL-10 (mDC/IL10) were generated, with or without IFN- γ activation, as in Figs. 2B-2D. In Fig. 2F allogeneic MLRs using enriched IDO $^+$ DCs (nonadherent cells, SFM system, without added IFN- γ). DCs were either immature or matured with antibody to CD40. APC/T cell ratio was 1:20. White bars, without 1MT; black bars, with 1MT. Fig. 2G presents MLR 30 with a titration of enriched IDO $^+$ DCs (nonadherent cells, SFM system, no IFN- γ) matured with cytokine-containing supernatant from activated monocytes. Similar results were observed when

DCs were matured with the cytokine regimen used in Fig. 1B. Responder T cell number, 5 X 10⁵ (highest APC/T cell ratio was 1:10) without 1MT (triangles), and with 1MT (squares). Fig. 2H represents immunomagnetic sorting of CD123⁺ DCs from a mixed DC preparation (BCS system, tumor necrosis factor- α (TNF- α) matured, no IFN- γ). Unfractionated (pre-sort), sorted 5 CD123⁺ cells (greater than 80% purity), and CD123-depleted cells. APC/T cell ratio was 1:10. White bars, with 1MT (DL-racemic mixture); black bars, without 1MT. Fig. 2I represents adherent cells (less than 10% IDO⁺) from SFM cultures, matured with TNF α /IL1 β /IL6/prostaglandin E₂, used as stimulators in allogeneic MLRs. White squares are without 1MT, black squares are with 1MT. For comparison, nonadherent (IDO⁺) cells from the 10 same culture are shown without 1MT (triangles). Representative of six experiments.

Figures 3A-3E. Validation of polyclonal anti-IDO antibody. Fig. 3A presents THP1 cell lysates, analyzed by western blot for IDO (12% SDS-PAGE gel, reducing conditions, blotted to PVDF membrane, blocked with 10% dry milk in 0.25% Tween-20 Tris-buffered saline). In lane 1, anti-IDO primary antibody (10 ng/ml) was detected with anti-rabbit peroxidase secondary 15 antibody (1:2000, Santa Cruz Biotechnology) and visualized by chemiluminescence (ECL, Amersham). Lane 2, as in lane 1, but with 1 ug/ml immunizing peptide added to the primary antibody before use. Fig. 3B presents MCSF-derived M ϕ s \pm IFN γ x 18 hours, analyzed by western blot as in Fig. 3A. Fig. 3C represents immunoprecipitation from THP1 cell lysates. Cells were lysed with buffer (150 mM NaCl, 0.5% NP-40, 1 mM dithiothreitol, 2 mM EDTA, 50 20 mM Tris pH 7.5, plus inhibitors of proteases and phosphatases) and lysates incubated with anti-IDO antibody (1 ug, lane 1), or antibody plus immunizing peptide (lane 2). Immune-complexes were precipitated with protein G-agarose (Life Technologies), resolved by SDS-PAGE under reducing conditions, and analyzed by silver stain. A single specific 45 kD band (arrow) was resolved (just below the heavy chain band of the immunoprecipitating antibody, IgGH). Fig. 3D 25 presents 2D-gel electrophoresis followed by western blotting with anti-IDO antibody revealed a single major immunoreactive species in resting M ϕ s (left panel). Following activation with IFN γ for 18 hours (right panel) two additional species (arrows) were detected by the anti-IDO antibody. MCSF-derived M ϕ s were lysed with 8M urea and 4% CHAPS in Tris buffer, and 50 ug of protein subjected to first-dimension isoelectric focusing (Protean IEF system, BioRad) 30 using pH 3-10 ampholyte strips. Second-dimension reducing SDS-PAGE was performed (Mini-Protean system, BioRad) and gels were transferred to PVDF membrane and analyzed by western

5 blot using anti-IDO antibody, as in Fig. 3A. Fig. 3E presents flow-cytometric analysis of MCSF-derived M ϕ s activated with IFN γ for 18 hours. Cells stained with anti-IDO antibody are shown on the left, while the same cells stained with antibody pre-adsorbed with the immunizing peptide are shown on the right.

10 Figures 4A-4B. Simultaneous analysis of T cell proliferation by thymidine incorporation and tryptophan concentration in the medium from the same allogeneic MLR culture. Fig. 4A presents an analysis of T cell proliferation by thymidine incorporation from the allogeneic MLR culture (day 3 of activation). Fig. 4B presents an analysis of tryptophan concentration in the medium by HPLC from the allogeneic MLR culture (day 3 of activation). Stimulators were IDO+ (nonadherent) DCs from SFM cultures. The ratio of DCs to T cells was low (1:500) in order to approximate the density of APCs expected in normal lymphoid tissue. Under these condition there is little depletion of tryptophan from the medium (starting tryptophan concentration in RPMI medium of 25 uM), and the concentration of tryptophan remains well above the level required to support T cell proliferation, indicated by the arrow in Fig. 4B.

15 Nevertheless, there was still a significant component of IDO-mediated suppression, shown by the 3-fold enhancement of proliferation seen when 1MT was added (Fig. 4A).

20 Figures 5A-5D. Suppression of T cell responses by tumor-draining LN cells. Fig. 5A shows dominant suppressor cells in TDLNs. Cells from tumor-draining LN (DLN) and contralateral LNs (CLN) were harvested from mice with B78H1.GMCSF tumors on day 14 and used as stimulators in MLRs (the number of stimulator cells is shown in parentheses). Responder cells (5×10^4 BM3 T cells) were the same in all groups. The left panel shows the absence of response when DLN cells were used as stimulators. The right panel shows mixing experiments, revealing that the absence of response to the DLN cells was due to a dominant suppressor activity present in these cells. In all experiments, response to CLN stimulators was quantitatively comparable to control stimulators (normal LN cells from non-tumor-bearing mice). Fig. 5B shows suppressive pDCs and Tregs in tumor-draining LNs. TDLN cells were sorted by 4-color flow cytometry into CD11c $^+$ B220 $^+$ pDCs (1-2%) and CD4 $^+$ CD25 $^+$ Tregs (2-3%), plus a third population of all other cells (95-97%). Each fraction was used as stimulator cells in parallel MLRs, using the number of cells that would have been present in 5×10^4 of the original TDLN population. All MLRs received 5×10^4 BM3 responders. Fig. 5C shows suppression by pDCs is mediated by IDO. TDLN cells were sorted into pDCs, Tregs, and the

non-suppressive “all other” cells, as in the previous panel (Tregs were discarded). Each population was used as stimulators for BM3 responders, in duplicate MLRs with and without the IDO inhibitor 1MT. The left panel shows TDNL cells from a normal (IDO-sufficient) host show dominant suppression by pDCs that is reversed by 1MT (arrows). The right panel shows tumor-draining LN cells from IDO-knockout host, showing no suppression, and no effect of 1MT. Fig. 5D shows excess L-tryptophan (250 uM, 10x) abrogates suppression by pDCs in a fashion comparable to 1MT. Experimental design, as in previous panel. There was no effect of 10x L-tryptophan when TDNL cells were derived from IDO-knockout mice.

Figures 6A-6C. Adoptive transfer of DCs from TDNLs creates profound local

10 immunosuppression in naive hosts. Fig. 6A shows antigen-driven recruitment of BM3 T cells into the LNs draining the site of adoptive transfer. CD11c⁺ DCs were purified from TDNLs (C57BL/6 hosts) and injected subcutaneously into naive CBA mice. Recipient mice had previously received 4×10^7 naive BM3 splenocytes injected intravenously (“CBA+BM3” mice). Control CBA+BM3 recipients received normal CD11c⁺ DCs prepared from LNs of C57BL/6 mice without tumors (“Normal antigen(+)”), or from LNs of syngeneic CBA mice (“Antigen(-)”). After 10 days, the LNs draining the site of the DC injection were harvested and stained for clonotype-specific BM3 TCR expression (left panel). Spleens from each animal were similarly stained (right panel). The number of clonotype-positive CD8⁺ cells, expressed as a percentage of total CD8⁺ T cells, are shown for each of the 3 different DC priming groups.

20 Each bar represents 4 pooled nodes. The absolute number of LN and spleen cells was not significantly different between the 3 groups. Fig. 6B shows unresponsiveness of T cells primed with DCs from TDNLs. Recipient CBA+BM3 mice were primed with the 3 different types of DCs described in Fig. 6A. After 10 days, LN cells draining the site of DC injection were harvested and used as responder cells in recall MLRs. A fixed number of the primed responder 25 cells from each group (1×10^5) were tested against a titration of stimulator cells (irradiated normal C57BL/6 splenocytes), as shown. In Fig. 6C recipient CBA+BM3 mice were primed with DCs from TDNLs, or with DCs from normal C57BL/6 LNs. During the 10 day priming exposure, half of the recipient mice received 1-methyl-D-tryptophan (5 mg/day) via implantable pellets; the group not labeled as receiving 1MT received vehicle pellets alone. After 10 days, 30 responder T cells were harvested from LNs draining the site of each DC injection, and tested for responsiveness in recall MLRs against a titration of irradiated C57BL/6 splenocytes. All

recipients were pre-loaded with identical aliquots of BM3, and all recall MLRs were performed in parallel with identical stimulators, so that comparison would be meaningful between groups.

Figures 7A-7C. Response to antigen introduced on IDO+ DCs. Fig. 7A shows systemic awareness of antigen introduced on TDNL DCs is revealed by TCR down regulation in spleen.

5 The upper panel represents in vitro stimulation of BM3 T cells by cells from TDNL caused down-regulation of TCR on BM3 T cells (detected by clonotype-specific antibody, gated on CD8+ cells). TCR down regulation was not induced by normal C57BL/6 LN stimulators. The lower panels represent adoptive transfer of CD11c⁺ DCs was performed as in Figure 6A. After 10 days, LNs and spleen were harvested from recipient CBA+BM3 mice stained for BM3 10 clonotype-specific TCR expression. The level of TCR expression is shown, gated on the clonotype-positive CD8⁺ BM3 cells in LN and spleen. Each histogram represents 4 pooled samples. Fig. 7B demonstrates TCR down regulation is dependent on functional IDO in the transferred DCs. B78H1 \times GM-CSF tumors were grown in IDO-knockout mice (C57BL/6 background). DCs were sorted from TDNLs and used to prime recipient CBA+BM3 mice; 15 control recipients received TDNL DCs from wild-type C57BL/6 hosts, or normal C57BL/6 DCs. In the left panel, TCR expression on CD8+ BM3 T cells (measured as in Fig. 7A) showed little down regulation in the absence of IDO. Representative data from recipient LNs are shown; comparable results were obtained with T cells from spleen. In other experiments, administration of 1MT at the time of adoptive transfer also significantly reduced TCR down regulation by 20 TDNL DCs. The right panel confirms that IDO-deficient TDNL DCs also did not suppress BM3 responses in recall MLRs. Fig. 7C represents creation of secondary, IDO-independent immunosuppression following adoptive transfer. CBA+BM3 recipients were primed with DCs from normal LNs (group #1), or with DCs from TDNLs (group #2), both from C57BL/6 mice. After 10 days, the recipient LNs draining the site of adoptive transfer were tested in recall MLRs. 25 In the left panel, mixing experiments showed that the lack of reactivity in T cells from mice receiving TDNL DCs was dominant, indicating active suppression. In the right panel, T cell proliferation was not restored by 1MT added to the MLR assays, suggesting that recipients had developed secondary, IDO-independent mechanisms of immunosuppression.

Figure 8. IDO-mediated suppressor activity segregates with the CD19⁺ subset of pDCs.

30 Cells from TDNLs were sorted into five populations based on expression of CD11c, B220, and CD19, as shown in the schematic at top. The three CD11c⁺ DC fractions (labeled “A,” “B,” and

“C”) were used as stimulators in MLRs. Control MLRs were stimulated by B cells from the same LN. Each MLR contained 2×10^5 BM3 responder cells, plus a titration of each type of stimulator cell (responders were in excess, stimulators were limiting). Parallel titrations were performed with and without 1MT, to demonstrate the IDO-mediated component of suppression.

5 Only the CD19⁺ pDCs (fraction A) mediated significant suppression.

Figures 9A-9D. The CD19⁺ DC subset displayed a phenotype consistent with mature plasmacytoid DCs. In Fig. 9A, TDLNs were stained by 4-color flow cytometry for CD11c versus CD19 versus the various markers shown. The total CD11c⁺ DC population was gated into CD19⁺ and CD19^{NEG} subsets, and the expression of each marker shown for the two populations.

10 The isotype-matched negative control for each marker (gated on CD11c⁺ cells) is shown as the horizontal bar. Each histogram is representative of 4-12 experiments with each marker. In Fig. 9B, cells from TDLNs were sorted into 5 fractions, as shown in Figure 8. RNA from each fraction was analyzed by real-time quantitative RT-PCR. RNA was added based on pre-determined equivalent amounts of gamma-actin message. Gels show the expected molecular weight band for CD19, pax5, and γ -actin after 27 cycles of amplification. The numbers below each band give the relative amount of message, quantitated against a standard curve of spleen RNA, normalized to the sorted B cell fraction (arbitrarily set equal to 100%). Fig. 6C represents expression of maturation markers on CD19⁺ versus CD19^{NEG} subsets of DCs from TDLNs, gated as in Fig. 9A. Maturation markers in the CD19^{NEG} population varied from experiment to experiment (an immature example is shown for comparison), but the CD19⁺ population was always mature. Fig. 9D represents expression of cell-surface markers CD123 (IL3R-a) and CCR6 on the CD19⁺ and CD19^{NEG} DC subsets. Cells were analyzed from TDLNs and from contralateral LNs of the same animals, as shown.

Figure 10. Superior inhibitory activity of the D isomer of 1MT in an in vitro bioassay.

25 The ability of D- and L-1MT to inhibit IDO-mediated suppression of human T cell proliferation was measured in allogeneic MLRs, using enriched IDO-expressing human DCs. Replicate MLRs received each compound at the concentrations shown. Bars represent T cell proliferation at the end of the 5-day activation period.

Figures 11A-11E. Administration of 1-MT enhances immune-mediated host anti-tumor

30 effect when administered with radiation or cyclophosphamide. Mice were injected subcutaneously (SQ) with 4×10^4 B16F10 cells. In Fig. 11A 1MT (20 mg/day of a DL racemic

mixture) was administered SQ by continuous-release copolymer pellets, with or without 500 cGy of total-body γ -irradiation. Control mice received vehicle pellets without drug. In Fig. 11B 1MT was administered with cyclophosphamide (CPM) (150 mg/kg, one dose). Fig. 11C represents 5 1MT and cyclophosphamide in rag1-knockout hosts. Fig. 11D represents the more potent pure D-isomer of 1MT, given 5 mg/day, with cyclophosphamide.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

A newly recognized molecular mechanism contributing to peripheral immune tolerance is the immunoregulatory enzyme indoleamine 2,3-dioxygenase (IDO). Cells expressing the 10 tryptophan-catabolizing enzyme IDO are capable of inhibiting T cell proliferation in vitro and reducing T cell immune responses in vivo (U.S. Patent Nos. 6,451,840 and 6,482,416; Munn et al., *Science* 1998;281:1191; Munn et al., *J. Exp. Med.* 1999;189:1363; Hwu et al., *J. Immunol.* 2000;164:3596; Mellor et al., *J. Immunol.* 2002;168:3771; Grohmann et al., *J. Immunol.* 2001;167:708; Grohmann et al., *J. Immunol.* 2001;166:277; and Alexander et al., *Diabetes* 15 2002;51:356).

IDO degrades the essential amino acid tryptophan (for reviews see Taylor et al., *FASEB Journal* 1991;5:2516-2522; Lee et al., *Laboratory Investigation*, 2003;83:1457-1466; and Grohmann et al., *Trends in Immunology* 2003;24:242-248). Expression of IDO by human monocyte-derived macrophages (Munn et al., *J. Exp. Med.* 1999;189:1363-1372), human 20 dendritic cells (Munn et al., *Science* 2002;297:1867-1870 and Hwu et al., *J. Immunol.* 2000;164:3596-3599), and mouse dendritic cells (Mellor et al., *J. Immunol.* 2003;171:1652-1655) allows these different antigen-presenting cells (APCs) to inhibit T cell proliferation in vitro. In vivo, IDO participates in maintaining maternal tolerance toward the antigenically foreign fetus during pregnancy (Munn et al., *Science* 1998;281:1191-1193).

IDO has also been implicated in maintaining tolerance to self antigens (Grohmann et al., *J. Exp. Med.* 2003;198:153-160), in suppressing T cell responses to MHC-mismatched organ transplants (Miki et al., *Transplantation Proceedings* 2001;33:129-130), and in the tolerance-inducing activity of recombinant CTLA4-Ig (Grohmann et al., *Nature Immunology* 2002;3:985-1109). In these three systems, the immunosuppressive effect of IDO can be blocked by the in 30 vivo administration of an IDO inhibitor, such as 1-methyl-tryptophan (also referred to herein as 1-MT or 1MT).

The transfection of IDO into mouse tumor cell lines confers the ability to suppress T cell responses both in vitro and in vivo (Mellor et al., *J. Immunol.* 2002;168:3771-3776). In a Lewis Lung carcinoma (LLC) model, administration of 1-MT significantly delayed tumor outgrowth (Friberg et al., *International Journal of Cancer* 2002;101:151-155). The mouse mastocytoma tumor cell line forms lethal tumors in naive hosts, but is normally rejected by pre-immunized hosts. However, transfection of P815 with IDO prevents its rejection by pre-immunized hosts (Uyttenhove et al., *Nature Medicine* 2003;9:1269-1274). This effect was entirely dependent on the presence of an intact immune system and was substantially reversed, that is, tumor growth inhibited, by the concomitant administration of 1-MT.

10 The selective recruitment of IDO⁺ APCs in the tumor-draining (sentinel) lymph nodes of patients with melanoma (Munn et al., *Science* 2002;297:1867-1870 and Lee et al., *Laboratory Investigation* 2003;83:1457-1466) indicates that tumors take advantage of the immunosuppressive effect of IDO by recruiting a population of IDO-expressing host APCs to present tumor antigens. Similar changes have been seen in breast carcinoma and other tumor-associated lymph nodes. In mouse tumor models the IDO-expressing APCs in tumor-draining lymph nodes are phenotypically similar to a subset of dendritic cells recently shown to mediate profound IDO-dependent immunosuppressive in vivo (Mellor et al., *Journal of Immunology* 2003;171:1652-1655). IDO-expressing APCs in tumor-draining lymph nodes thus constitute a potent tolerogenic mechanism.

20 The present invention is based on the observation that the administration of an inhibitor of indoleamine-2,3-dioxygenase to a subject suffering from a tumor or infection in combination with administration of an additional therapeutic agent, results in an improved efficacy of therapeutic outcome when compared to the therapeutic outcome observed with the administration of the inhibitor of indoleamine-2,3-dioxygenase alone or the administration of the 25 additional therapeutic agent alone. While not intending to be limited to any single mechanism, the improved efficacy of therapeutic outcome observed may be due to a removal, reversal, or reduction of the immunosuppressive effect of indoleamine-2,3-dioxygenase by the administration an inhibitor of indoleamine-2,3-dioxygenase.

30 In some embodiments of the present invention, the improved efficacy of therapeutic outcome observed with the administration of an inhibitor of indoleamine-2,3-dioxygenase in combination with an additional therapeutic agent may be demonstrated by determination of the

therapeutic synergy. As used herein, a combination manifests “therapeutic synergy” if it is therapeutically superior to one or other of the constituents used at its optimum dose (Corbett et al., *Cancer Treatment Reports*, 66, 1187 (1982). In some embodiments of the present invention, the efficacy of a combination may be characterized by adding the actions of each constituent.

5 The administration of both an inhibitor of indoleamine-2,3-dioxygenase and at least one additional therapeutic agent can result in an augmentation of the rejection of cells in a subject, wherein the rejection of cells obtained by administering both the inhibitor of indoleamine-2,3-dioxygenase and the additional therapeutic agent is greater than that obtained by administering either the inhibitor of indoleamine-2,3-dioxygenase or the additional therapeutic agent alone. As used herein the augmented rejection of cells includes an increased level of immune system mediated rejection of the cells. As used herein, “cell” can include tumor cells and cells infected with an intracellular pathogen.

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Likewise, the administration of both an inhibitor of indoleamine-2,3-dioxygenase and at least one additional therapeutic agent can result in an increased cancer survival rate, a reduced or 15 slowed tumor growth, the reduction in relapse to neoplasm, as in leukemia, a reduction in tumor progression, or a reduction in tumor metastasis, in comparison to that observed with the administration of either the inhibitor of indoleamine-2,3-dioxygenase or the additional therapeutic agent alone. As used herein, “increased cancer survival rate,” “reduced tumor growth,” “slowed tumor growth,” “reduced relapse to neoplasm,” “reduced tumor progression,” 20 or “reduced tumor metastasis” are as determined by established clinical standards.

The determination of immunosuppression mediated by an antigen presenting cell expressing indoleamine-2,3-dioxygenase (IDO) includes the various methods as described in the examples herein. T cell activation by an antigen-presenting cell and the stimulation of an immune response are as measured by standard methods well known in the immunological arts.

25 The enzyme indoleamine-2,3-dioxygenase (IDO) is well characterized (see, for example, Taylor et al., *FASEB Journal* 1991;5:2516-2522; Lee et al., *Laboratory Investigation*, 2003;83:1457-1466; and Grohmann et al., *Trends in Immunology* 2003;24:242-248). Compounds that serve as substrates or inhibitors of the IDO enzyme are also well known. For example, Southan et al., (*Med. Chem Res.*, 1996;343-352) utilized an *in vitro* assay system to 30 identify tryptophan analogues that serve as either substrates or inhibitors of human IDO.

IDO inhibitors of the present invention include, but are not limited to, 1-methyl-tryptophan, β -(3 benzofuranyl)-alanine, β -[3-benzo(b)thienyl]-alanine, 6-nitro-tryptophan, and derivatives thereof. Inhibitors of the IDO enzyme are readily commercially available, for example, from Sigma-Aldrich Chemicals, St. Louis, MO. An inhibitor of indoleamine-2,3-dioxygenase may be a L isomer of an inhibitor of indoleamine-2,3-dioxygenase, a D isomer of an inhibitor of indoleamine-2,3-dioxygenase, or a racemic mixture of an inhibitor of indoleamine-2,3-dioxygenase. In some embodiments, a preferred IDO inhibitor is 1-methyl-tryptophan, also referred to herein 1MT.

In accordance with the present invention, an IDO inhibitor is administered to a subject in combination with the administration of one or more previously known treatment modalities. As used herein, the term “additional therapeutic agent” represents one or more agents administered in the previously known treatment modality. An additional therapeutic agent is not an inhibitor of IDO. For example, inhibitors of IDO may be administered to a patient in combination with one or more other modes of cancer treatment. Such additional therapeutic agents include, but are not limited to, chemotherapy, radiation therapy, hormone therapy, surgical resection, treatment with an immunostimulatory cytokine, administration of an anti-tumor vaccine, antibody based therapies, whole body irradiation, bone marrow transplantation, and peripheral blood stem cell transplantation. The chemotherapeutic agents used include, but are not limited to, cyclophosphamide, methotrexate, fluorouracil, doxorubicin, vincristine, ifosfamide, cisplatin, gemcytabine, busulfan (also known as 1,4-butanediol dimethanesulfonate or BU), ara-C (also known as 1-beta-D-arabinofuranosylcytosine or cytarabine), adriamycin, mitomycin, cytoxan, methotrexate, and combinations thereof. The cytokines used include, but are not limited to, IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-6, IL-8, IL-9, IL-10, IL-12, IL-18, IL-19, IL-20, IFN- α , IFN- β , IFN- γ , tumor necrosis factor (TNF), transforming growth factor- β (TGF- β), granulocyte colony stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF) (U.S. Patent Nos. 5,478,556, 5,837,231, and 5,861,159), or Flt-3 ligand (Shurin et al., *Cell Immunol.* 1997; 179:174-184).

An IDO inhibitor may also be administered to a patient in combination with other modes of treatment for an infection. Such additional therapeutic agents may include, but are not limited to antiviral agents, antibiotics, antimicrobial agents, cytokines, and vaccines. The cytokines used include, but are not limited to, IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-6, IL-8, IL-9, IL-10, IL-12, IL-

18, IL-19, IL-20, IFN- α , IFN- β , IFN- γ , tumor necrosis factor (TNF), transforming growth factor- β (TGF- β), granulocyte colony stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF) (U.S. Patent Nos. 5,478,556, 5,837,231, and 5,861,159), or Flt-3 ligand (Shurin et al., *Cell Immunol.* 1997; 5 179:174-184).

An IDO inhibitor may be administered to a patient receiving a vaccine. Such a vaccine may be an anti-viral vaccine, such as, for example, a vaccine against HIV, or a vaccine against tuberculosis or malaria. The vaccine may be a tumor vaccine, including, for example, a melanoma, prostate cancer, colorectal carcinoma, or multiple myeloma vaccine. Dendritic cells (DC) have the ability to stimulate primary T cell antitumor immune responses. Thus, a tumor vaccine may include dendritic cells. Dendritic cell vaccines may be prepared, for example, by pulsing autologous DCs derived from the subject with synthetic antigens, tumor lysates or tumor RNA, or idiotype antibodies, or transfection of DCs with tumor DNA, or by creating tumor cell/DC fusions (Ridgway, *Cancer Invest.* 2003;21(6):873-86). The vaccine may include one or 10 more immunogenic peptides, for example, immunogenic HIV peptides, immunogenic tumor peptides, or immunogenic human cytomegalovirus peptides (such as those described in U.S. Patent No. 6,251,399). The vaccine may include genetically modified tumor cells, including genetically modified tumor cells to express granulocyte-macrophage stimulating factor (GM-CSF) (Dranoff, *Immunol Rev.* 2002;188:147-54).

20 The administration of the IDO inhibitor may take place before, during, or after the administration of the other mode of therapy.

Inhibitors of IDO may be formulated as a composition. The compositions of the present invention may be formulated in a variety of forms adapted to the chosen route of administration. The formulations may be conveniently presented in unit dosage form and may be prepared by 25 methods well known in the art of pharmacy. Formulations of the present invention include, for example, pharmaceutical compositions including an IDO inhibitor and a pharmaceutically acceptable carrier. The phrase "pharmaceutically-acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human. The preparation of such compositions is well understood in the art. The formulations of 30 this invention may include one or more accessory ingredients including diluents, buffers,

binders, disintegrants, surface active agents, thickeners, lubricants, preservatives (including antioxidants), and the like.

Formulations of an IDO inhibitor may further include one or more additional therapeutic agents. An additional therapeutic agent may be an antineoplastic chemotherapy agent, including, 5 but not limited to, cyclophosphamide, methotrexate, fluorouracil, doxorubicin, vincristine, ifosfamide, cisplatin, gemcytabine, busulfan (also known as 1,4-butanediol dimethanesulfonate or BU), ara-C (also known as 1-beta-D-arabinofuranosylcytosine or cytarabine), adriamycin, mitomycin, cytoxan, methotrexate, or a combination thereof. Additional therapeutic agents include cytokines, including, but not limited to, macrophage colony stimulating factor, interferon 10 gamma, granulocyte-macrophage stimulating factor (GM-CSF), flt-3, an antibiotic, antimicrobial agents, antiviral agents, including, but not limited to, AZT, ddI or ddC, and combinations thereof.

The tumors to be treated by the present invention include, but are not limited to, melanoma, colon cancer, pancreatic cancer, breast cancer, prostate cancer, lung cancer, 15 leukemia, lymphoma, sarcoma, ovarian cancer, Kaposi's sarcoma, Hodgkin's Disease, Non-Hodgkin's Lymphoma, multiple myeloma, neuroblastoma, rhabdomyosarcoma, primary thombocytosis, primary macroglobulinemia, small-cell lung tumors, primary brain tumors, stomach cancer, malignant pancreatic insulanoma, malignant carcinoid, urinary bladder cancer, premalignant skin lesions, testicular cancer, lymphomas, thyroid cancer, neuroblastoma, 20 esophageal cancer, genitourinary tract cancer, malignant hypercalcemia, cervical cancer, endometrial cancer, and adrenal cortical cancer. As used herein, "tumor" refers to all types of cancers, neoplasms, or malignant tumors found in mammals.

The efficacy of treatment of a tumor may be assessed by any of various parameters well known in the art. This includes, but is not limited to, determinations of a reduction in tumor size, 25 determinations of the inhibition of the growth, spread, invasiveness, vascularization, angiogenesis, and/or metastasis of a tumor, determinations of the inhibition of the growth, spread, invasiveness and/or vascularization of any metastatic lesions, and/or determinations of an increased delayed type hypersensitivity reaction to tumor antigen. The efficacy of treatment may also be assessed by the determination of a delay in relapse or a delay in tumor progression in the 30 subject or by a determination of survival rate of the subject, for example, an increased survival

rate at one or five years post treatment. As used herein, a relapse is the return of a tumor or neoplasm after its apparent cessation, for example, such as the return of a leukemia.

Certain pathological conditions, such as parasitic infections, AIDS (caused by the human immunodeficiency virus (HIV) and latent cytomegaloviral (CMV) infections, are extremely difficult to treat since the macrophages act as reservoirs for the infectious agent. Even though the cells are infected with by a foreign pathogen, they are not recognized as foreign. The methods of the present invention may be used to treat such pathological conditions including, but not limited to, viral infections, infection with an intracellular parasite, and infection with an intracellular bacteria. Viral infections treated include, but are not limited to, infections with the human immunodeficiency virus (HIV) or cytomegalovirus (CMV). Intracellular bacterial infections treated include, but are not limited to infections with *Mycobacterium leprae*, *Mycobacterium tuberculosis*, *Listeria monocytogenes*, and *Toxoplasma gondii*. Intracellular parasitic infections treated include, but are not limited to, *Leishmania donovani*, *Leishmania tropica*, *Leishmania major*, *Leishmania aethiopica*, *Leishmania mexicana*, *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, and *Plasmodium malariae*.

The efficacy of treatment of an infection may be assessed by any of various parameters well known in the art. This includes, but is not limited to, a decrease in viral load, an increase in CD4⁺ T cell count, a decrease in opportunistic infections, eradication of chronic infection, and/or increased survival time.

An inhibitor of IDO may be administered to a subject following bone marrow transplantation or a peripheral blood stem cell transplantation. The efficacy of such an administration may be assessed by any of a variety of parameters well known in the art. This includes, for example, determinations of an increase in the delayed type hypersensitivity reaction to tumor antigen, determinations of a delay in the time to relapse of the post-transplant malignancy, determinations of an increase in relapse free survival time, and/or determinations of an increase in post-transplant survival. The IDO inhibitor may be administered to the subject prior to full hematopoietic reconstitution or prior to recovery from lymphopenia.

The inhibitors of the present invention can be administered by any suitable means including, but not limited to, for example, oral, rectal, nasal, topical (including transdermal, aerosol, buccal and sublingual), vaginal, parenteral (including subcutaneous, intramuscular, intravenous and intradermal), intravesical, or injection into or around the tumor.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous, intraperitoneal, and intratumoral administration. In this connection, sterile aqueous media that can be employed will be known to those of skill in the art in light of the present disclosure (see for example, "Remington's Pharmaceutical Sciences" 15th Edition). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, and general safety and purity standards as required by the FDA.

For enteral administration, the inhibitor will typically be administered in a tablet or capsule, which may be enteric coated, or in a formulation for controlled or sustained release. Many suitable formulations are known, including polymeric or protein microparticles encapsulating drug to be released, ointments, gels, or solutions which can be used topically or locally to administer drug, and even patches, which provide controlled release over a prolonged period of time. These can also take the form of implants. Such implant may be implanted within the tumor.

Therapeutically effective concentrations and amounts may be determined for each application herein empirically by testing the compounds in known *in vitro* and *in vivo* systems, such as those described herein; dosages for humans or other animals may then be extrapolated therefrom.

An IDO inhibitor may be administered at once, or may be divided into a number of smaller doses to be administered at intervals of time. It is understood that the precise dosage and duration of treatment is a function of the disease being treated and may be determined empirically using known testing protocols or by extrapolation from *in vivo* or *in vitro* test data. It is to be noted that concentrations and dosage values may also vary with the severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the

compositions, and that the concentration ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed compositions and methods.

The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

EXAMPLES

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Example 1

Potential Regulatory Function of Human Dendritic Cells Expressing Indoleamine 2,3-Dioxygenase

This example describes a subset of human APCs that express indoleamine 2,3-dioxygenase (IDO) and inhibit T cell proliferation in vitro. IDO-positive APCs constituted a discrete subset identified by coexpression of the cell-surface markers CD123 and CCR6. In the dendritic cell (DC) lineage, IDO-mediated suppressor activity was present in fully mature as well as immature CD123⁺ DCs. IDO⁺ DCs could also be readily detected in vivo, indicating that these cells represent a regulatory subset of APCs in humans.

Using an IDO-specific antibody (Fig. 3), it was shown by flow cytometry that fresh human monocytes expressed low to undetectable levels of the IDO protein (Fig. 1A). Monocyte-derived macrophages (Mφs) up-regulated IDO upon activation with interferon- γ (IFN- γ) (Munn et al., *J. Exp. Med.*, 1999; 189:1363). Expression of IDO in these cells was confined to a particular subset of cells coexpressing CD123 (the interleukin-3 (IL-3) receptor α chain] and the chemokine receptor CCR6 (Fig. 1A). Similarly, monocyte-derived DCs (Sallusto and Lanzavecchia, *J. Exp. Med.*, 1994; 179:1109) expressed IDO, which was also confined to a CD123⁺, CCR6⁺ subset.

Because serum factors are known to influence DC maturation (Romani et al., *J. Immunol. Methods*, 1996; 196:137), DCs were derived in both bovine calf serum (BCS)-based medium and serum-free medium (SFM). Both systems yielded IDO⁺ DCs with the same phenotype, but greater than 90% of the IDO^{NEG} cells in SFM were tightly adherent. This allowed facile

enrichment of the nonadherent IDO⁺ population to greater than 90% purity. The IDO⁺ cells expressed cell-surface markers CD14^{NEG}, CD83⁺, CD80⁺, CD86^{HI}, and HLA-DR^{HI} (Fig. 1B), morphology consistent with mature DCs (Fig. 1C). Adherent cells lacked CD83 and displayed residual levels of CD14, consistent with an immature or transitional phenotype. Although IDO⁺ 5 cells in DC cultures expressed DC-specific lineage markers, and the IDO⁺ Mφs expressed Mφ-lineage markers (Fig. 1D), in both types of cells the IDO⁺ subset could be specifically identified by expression of CD123 and CCR6.

As shown previously (Munn et al., *J. Exp. Med.*, 1999; 189:1363), resting macrophage colony-stimulating factor (MCSF)-derived Mφs did not express high concentrations of IDO until 10 they received a triggering signal such as IFN-γ (Fig. 2A). In contrast, IDO could be detected constitutively in CD123⁺ DCs (Fig. 2B). However, activation with IFN-γ was still required for expression of functional enzymatic activity Fig. 2E), which suggests that the IDO protein could exist in both enzymatically active and inactive states. Posttranslational regulation of enzymatic activity (constitutive expression of enzyme protein but with additional signals being required for 15 functional activity) is a feature of many regulatory enzymes. Expression of IDO protein without enzymatic activity has been described in murine DCs (Fallarino et al., *Int. Immunol.*, 2002;14:65). However, the mechanism by which IDO might exist in distinct functional states remains to be determined. Because the maturational status of DCs may affect a number of functional attributes of these cells, it was determined whether maturation affected IDO 20 expression by CD123⁺ DCs. Although maturation itself had no effect on the constitutive (basal) expression of IDO protein, subsequent activation of mature DCs with IFN-γ resulted in complete down-regulation of IDO. This was a consistent observation in 16 experiments with 10 different donors and was confirmed by flow cytometry (Fig. 2C), enzymatic activity (Fig. 2E), and mRNA.

25 Interleukin 10 (IL-10) is a regulatory cytokine that has been associated with the development of tolerogenic DCs (Steinbrink et al., *J. Immunol.*, 1997;159:4772). The presence of IL-10 during maturation prevented IFN-γ-induced down-regulation of IDO, resulting in sustained expression of functional IDO even in mature, IFN-γ-activated DCs (Figs 2D and 2E). Similar results were observed when transforming growth factor-β was present during maturation. 30 Taken together, these data raised the possibility that expression of IDO by mature DCs might be determined by the prevailing regulatory influences during maturation.

DCs were next tested for their ability to stimulate T cells in allogeneic mixed-leukocyte reactions (MLRs). In Figs. 2B-2E, recombinant IFN- γ was added to simulate signals from activating T cells (Munn et al., *J. Exp. Med.*, 1999 189:1363), but for MLRs the DCs received no exogenous IFN- γ . Immature DCs, selected and enriched to >90% purity for IDO expression 5 (Fig. 1B), stimulated very little T cell proliferation (Fig. 2F). However, in most donors proliferation could be significantly enhanced by addition of 1-methyl-D-tryptophan (1MT), a competitive inhibitor of IDO. After maturation, enriched IDO $^+$ DCs displayed one of two patterns: in 4 of 45 experiments the mature DCs lost their IDO-mediated inhibitory activity (Fig. 2F), whereas in 41 of 45 experiments they maintained potent inhibitory activity despite 10 maturation, which was reversed by the addition of 1MT (Fig. 2G). Continued expression of IDO by mature DCs in the latter experiments was confirmed by flow cytometry on MLR cultures and by measurement of tryptophan and kynurenine in supernatants. The two different patterns observed in MLR were suggestive of the different patterns observed when mature DCs were tested in isolation (Fig. 2C and 2D).

15 The experiments in Fig. 2G were performed with highly enriched IDO $^+$ DCs from SFM cultures. In contrast, BCS medium yielded a mixture of nonadherent IDO $^+$ and IDO $^{\text{NEG}}$ cells, with IDO $^{\text{NEG}}$ cells typically in the majority. Under these conditions, T cell activation predominated, and 1MT had little detectable effect (Fig. 2H). However, when the IDO $^+$ DCs 20 were enriched from such mixtures by sorting for CD123 expression, they displayed inhibitory activity comparable to the IDO $^+$ DCs from SFM (Fig. 2H). To verify the specificity of 1MT as an inhibitor of IDO, 1MT was added to MLRs containing APCs that did not express inhibitory amounts of IDO (adherent cells from SFM cultures, less than 10% IDO). Under these 25 conditions, T cell proliferation was not inhibited, and 1MT had no effect on T cell proliferation (Fig. 2I).

25 The staining of tissue from fifteen non-inflamed tonsils removed in routine tonsillectomy showed scattered IDO $^+$ cells in germinal center and T cell regions, indicating that *in vivo*, few IDO $^+$ cells are detected in normal lymphoid tissue. However, human tonsils displaying features of chronic inflammation often possessed intense focal infiltrates of IDO $^+$ cells, which were 30 morphologically distinct from Ham56 $^+$ macrophages (Orenstein and Wahl, *Ultrastruct. Pathol.*, 1999;23:79) or S100 $^+$ interdigitating DCs (Cochran et al., *Mod. Pathol.*, 2001;14:604). Some IDO $^+$ cells coexpressed CD83, a marker of mature DCs, and some expressed CD123 and CCR6

(Fig. 3B). However, not all CD83⁺ (or CD123⁺ or CCR6⁺) cells expressed IDO, and no single marker identified all IDO⁺ cells, which suggests that IDO may be expressed by more than one population in vivo. Analyses of regional and sentinel (first draining) lymph nodes taken from patients with malignant melanoma revealed that 25 of 66 patients had one or more nodes with 5 abnormal accumulation of IDO⁺ cells.

For these studies, lymph nodes were analyzed from 26 patients with malignant melanoma at the Medical College of Georgia; 13 of 26 patients were found to have at least one node (often more than one) with markedly abnormal accumulation of IDO⁺ cells (3+ or 4+ on a 4+ scale, independently graded by 3 pathologists). The IDO⁺ cells were morphologically monocytic or 10 plasmacytoid in appearance, and were found infiltrating extensively throughout the lymphoid regions, typically concentrating in the interfollicular and T cell zones. Accumulations were observed around blood vessels, at the margins of medullary sinuses, and the interface between lymphoid tissue and tumor metastases. Recruitment of IDO⁺ dendritic cells to specific regional 15 lymph nodes was also seen in smaller series of patients with carcinoma of the breast, lung, colon and pancreas (comprising all of the other histologies examined).

In a separate study, sentinel lymph nodes were obtained from patients with malignant melanoma. Each node was documented to be the initial tumor-draining lymph node by in vivo lymphoscintigraphy, and all were negative for metastatic tumor by conventional pathologic studies. Twelve of these 40 sentinel nodes were found to have abnormal accumulation of IDO⁺ 20 cells (grades 1+ to 4+). In many of these patients, accumulation of IDO⁺ cells in the sentinel node occurred before overt metastasis. Similar accumulation of IDO⁺ cells was found in nodes from patients with breast, colon, lung, and pancreatic cancers.

In this example, a subset of human monocyte-derived DCs that use IDO to inhibit T cell proliferation in vitro is described. In both DC and M lineages, IDO⁺ cells could be characterized 25 by coexpression of CD123 and CCR6 (despite the expression of otherwise distinct lineage-specific markers), which suggests that the IDO⁺ population may represent a discrete subset of professional APCs. IDO⁺ DCs expressed major histocompatibility complex class II and costimulatory molecules and were effective stimulators of T cell proliferation when IDO was blocked by 1MT, which suggests that these cells could act as competent APCs. This may reflect 30 a regulatory subset of APCs specialized to cause antigen-specific depletion (Munn et al., *J. Immunol.*, 1996;156:523) or otherwise negatively regulate the responding population of T cells.

In light of the finding that large numbers of such cells are present in a proportion of tumor-draining lymph nodes, it appears that IDO⁺ APCs may participate in the state of apparent immunologic unresponsiveness displayed by many cancer patients toward tumor-associated antigens. However, the extent to which IDO-expressing APCs might influence immunologic unresponsiveness *in vivo* remains to be determined.

Polyclonal antibody preparation and validation

The peptide LIESGQLRERVEKLNMLC (SEQ ID NO:1) was prepared based on the GenBank sequence of human IDO (GenBank Accession Number M34455; Dai and Gupta, 10 *Biochem. Biophys. Res. Commun.*, 1990;168:1-8) and conjugated to keyhole limpet cyanogen. Rabbits were immunized with conjugated peptide in complete Freund's adjuvant and boosted three times in incomplete Freund's adjuvant (all antibody preparation and affinity purification steps were performed by QCB/BioSource International). This peptide gave the best results out of several sequences screened for their ability to detect IDO in formalin-fixed paraffin-embedded 15 tissue and flow cytometry. Using THP1 monocytic leukemia cell lysates, the antibody detected a single band of the expected 45 kD molecular mass (Dai and Gupta, *Biochem. Biophys. Res. Commun.*, 1990;168:1-8) by western blot (Fig. 3A). Immunoreactivity in western blot was blocked by pre-adsorption of the antibody with an excess of the immunizing peptide (Fig. 3A). The 45 kD band was inducible by IFN γ in human monocyte-derived M ϕ s (Fig. 3B), consistent 20 with previous western blot studies by others (Thomas et al., *J. Immunol.*, 2001;166:6332; and Grant et al., *J. Virol.*, 2000;74:4110). Immunoprecipitation of THP1 cell lysates yielded a single 45 kD protein band on silver stain, which was not precipitated in the presence of the immunizing peptide (Fig. 3C). The antibody detected an IFN γ -inducible antigen by flow cytometry in 25 monocyte-derived macrophages, and this signal was reduced by greater than 95% by pre-adsorption of the antibody with the immunizing peptide (Fig. 3D). By immunohistochemistry on formalin-fixed, paraffin-embedded specimens of human placenta, the antibody detected an antigen specifically localized to syncytiotrophoblast (Fig. 3E). Syncytiotrophoblast was used for validation because it is an unambiguous cell type that has been previously shown to express IDO by immunohistochemistry (Kamimura et al., *Acta. Med. Okayama*, 1991;45:135), which has 30 been independently confirmed using enzymatic-activity assays on highly purified placental cell fractions (Kudo and Boyd, *Biochem. Biophys. Acta*, 2000;1500:119). Reactivity in

immunohistochemistry was fully blocked by pre-adsorption of the antibody with the immunizing peptide (Fig. 3E).

Distinction of IDO⁺ cells from CD123⁺ plasmacytoid DCs

CD123 is also found on “plasmacytoid” DCs or pre-DC2 cells. However, the CD123 expression observed on the IDO⁺ subset of monocyte-derived DCs was at the lower level described on myeloid DCs (Cella et al., *Nat. Med.*, 1999;5:919), not the 10- to 100-fold higher levels that we observed on plasmacytoid DCs. The IDO⁺ cells also expressed myeloid markers (CD11b, CD11c) and did not express the pre-DC2 marker BDCA2 (Dziona et al., *J. Immunol.*, 2000;165:6037). Plasmacytoid dendritic cells (CD123⁺ CD11c^{NEG} cells) showed no detectable expression of IDO (fresh or following activation with IFN γ). The CD123⁺ IDO⁺ subset of monocyte-derived DCs and M ϕ s co-expressed CCR6. While CD123 was expressed constitutively, CCR6 was inducible, and was expressed under the same conditions in which IDO protein was induced. Thus, in MCSF-derived M ϕ s CCR6 required induction by IFN γ , whereas in DCs CCR6 was constitutively expressed on CD123⁺ DCs.

Monocyte isolation and culture

Human monocytes (typically greater than 95% purity) were isolated by leukocytapheresis and counterflow elutriation as described (Munn et al., *J. Exp. Med.*, 1999;189:1363), then cryopreserved in replicate aliquots. Monocyte-derived M ϕ s were cultured in RPMI-1640 medium supplemented with 10% bovine calf serum (Hyclone) (“BCS system”) and received MCSF (200 units/ml, gift of Genetics Institute) on day 0 (Munn et al., *J. Exp. Med.*, 1999;189:1363). Monocyte-derived DCs were cultured in 100 mm petri dishes in either BCS medium (Sallusto and Lanzavecchia, *J. Exp. Med.*, 1994;179:1109) or in serum-free medium (X-vivo 15, BioWhitaker, “SFM system”) (Chen et al., *Blood*, 1998;91:4652). SFM yielded a somewhat higher proportion of IDO⁺ cells, but the phenotype and function of the cells was identical. DCs received GMCSF (50 ng/ml, R&D Systems) + IL4 (50 ng/ml, R&D Systems) on days 0, 2 and 4. For experiments where CCR6 expression was of interest, cultures received a single dose of GMCSF + IL4 (100 ng/ml each) on day 0, which gave higher expression of CCR6 (Yang et al., *J. Immunol.*, 1999;163:1737). Non-adherent DCs were harvested by aspiration; adherent cells and MCSF-derived macrophages were harvested with 5 mM EDTA. For

maturation studies, cells were treated for the final 48 hours with one of the following: 0.5 ug/ml activating anti-CD40 antibody (Mabtech); a cytokine cocktail comprising TNF α (1100 units/ml, Pharmingen), IL1 β (1870 units/ml, Pharmingen), IL6 (1000 units/ml, Pharmingen) and PGE2 (1 ug/ml, Sigma) (Jonuleit et al., *Eur. J. Immunol.*, 1997;27:3135) (12); or 50% v/v conditioned 5 medium (X-vivo 15) from activated monocytes (allowed to adhere overnight to petri dishes coated with immobilized IgG (Cappel ICN) (Reddy et al. *Blood*, 1997;90:3640). In some experiments, DCs also received IL10 (R&D Systems, 100 ng/ml) with the maturation stimuli. For activation studies, cells received 100 units/ml IFN γ (Genentech) during the final 18 hours of culture.

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Flow cytometry

All antibodies and reagents were from BD-Pharmingen unless specified. Fresh whole blood was stained using BD FACS Lysing Solution. For cultured cells, nonadherent cells were pooled with adherent cells (harvested with 5 mM EDTA in PBS for 10 minutes) prior to analysis, 15 unless otherwise specified. Cells were triple-stained with anti-CD123-biotin (clone 7G3) followed by streptavidin-perCP, plus anti-CD11c-allophycocyanin (clone S-HCL-3) or anti-CCR6-fluorescein (clone 53103.111, R&D systems). CCR6 results were confirmed using a second antibody (11A9, BD-Pharmingen). After fixation and permeabilization (Cytofix/Cytoperm), cells were stained with rabbit anti-IDO antibody followed by PE-labeled 20 anti-rabbit secondary antibody (Jackson Immunoreasearch, cross-adsorbed against mouse, human and bovine IgG). For all experiments, the negative control for IDO staining was the anti-IDO antibody pre-adsorbed with a 50-fold molar excess of the immunizing peptide. Dendritic cells and M ϕ s were gated on forward and side scatter to exclude contaminating lymphocytes and debris. For phenotyping experiments, cells were stained without permeabilization, using a 25 multicolor panel of CD123 versus various markers (all from BD-Pharmingen).

Mixed-leukocyte reactions

Dendritic cells were mixed with 5×10^5 allogeneic lymphocytes (80-85% T cells, balance B cells and NK cells, less than 1% monocytes) in 250 ul of MLR culture medium (10% fetal calf serum in RPMI-1640). Replicate wells received 1-methyl-tryptophan (Aldrich) at a final 30 concentration of 200 uM at the time of T cell addition, or buffer control. Unless otherwise specified, the D-isomer of 1MT was used. After five days, proliferation was measured by four

hour thymidine incorporation assay. “V”-bottom culture wells (Nunc) were used in MLR assays to maximize cell-cell contact. It was found that this geometry gave significantly greater sensitivity for measuring suppression, as compared to flat-bottom wells. However, V-bottom wells were not a prerequisite for detecting suppression (e.g., all of the experiments in both Munn 5 et al., *J. Exp. Med.*, 1999; 189:1363; and Mellor et al., *J. Immunol.*, 2002; 168:3771 used flat-bottom wells). The V-bottom geometry itself was not suppressive, as shown by the brisk T cell proliferation observed when IDO was inhibited by 1MT (Fig. 2G), or when IDO^{NEG} APCs were used as stimulators (Fig 2I). Tryptophan depletion in these cultures is shown in Fig. 4. The data represented in Figure 4 indicate that inhibition of proliferation did not depend on artificially high 10 concentrations of APCs or global depletion of tryptophan from the entire culture medium. However, these experiments were still consistent with a localized depletion of tryptophan (i.e., occurring within the immediate zone of contact between DCs and T cells), since addition of supra-physiologic levels of tryptophan to the culture medium (250 uM) completely reversed the DC-mediated inhibition of T cell proliferation, as has been previously described (Munn et al., *J. 15 Exp. Med.*, 1999; 189:1363).

Immunohistochemistry

For paraffin blocks, sections were deparaffinized, treated for 8 minutes with proteinase K (Dako), and stained with rabbit anti-human IDO antibody (5 ug/ml in phosphate-buffered saline 20 with 0.05% Tween-20 and 10% goat serum). Detection was via secondary antibody conjugated to alkaline phosphatase (LSAB2-rabbit kit, Dako) with Fast Red or nitro-blue tetrazolium chromogen. In all experiments, negative controls consisted of the anti-IDO antibody neutralized with a molar excess of the immunizing peptide. For two-color immunofluorescent staining, frozen sections of human tonsil were fixed for 10 minutes in 10% formalin, then primary 25 antibodies were applied against IDO (0.05 ug/ml) and either CD83 (Immunotech), CD123 (Santa Cruz) or CCR6 (R&D Systems). IDO was detected with rabbit-specific secondary antibody conjugated to Alexa-488 (Molecular Probes); mouse antibodies were detected with species-specific secondary antibody conjugated to Alexa-568 (all secondary antibodies were cross-adsorbed for multiple labeling). Some CD4⁺ T cells showed low-level staining for IDO, 30 consistent with previous reports (Curreli et al., *J. Interferon Cytokine Res.*, 2001; 21:431). This T cell staining was minimal by the less-sensitive immunohistochemistry technique, and with

immunofluorescence could be unambiguously distinguished from the high-level IDO expression seen in APCs through the use of two-color staining.

Use of recombinant IFN γ

5 When DCs or M ϕ s were tested in isolation (i.e., without T cells, as in Figs. 2A-2E) recombinant human IFN γ was added during the final 18 hours of culture), in order to simulate the activating signals that would normally be delivered by activating T cells to APCs during antigen-presentation (Munn et al., *J. Exp. Med.*, 1999;189:1363). This allowed the identification of 3 stages at which IDO expression could be regulated in DCs; differentiation, maturation, and
10 the final activation step (e.g., by IFN γ). Simulating this final step proved critical in elucidating the differential regulation of IDO in mature versus immature DCs (see Figs. 2B-2D). In this system, IFN γ was found to be capable of both up-regulating and down-regulating IDO, depending the maturation status of the DCs. This dual role for IFN γ is consistent with the literature, which has shown roles for IFN γ both as a pro-inflammatory cytokine, and as a
15 participant in tolerance and negative regulation (Konieczny et al., *J. Immunol.*, 1998;160:2059). When DCs were to be used in MLRs they were not exposed to IFN γ during culture, nor was recombinant IFN γ added to the MLRs. Thus, any activating signals influencing expression of IDO in MLRs would be derived physiologically, from the T cells themselves.

20 *Two patterns of IDO expression in mature DCs*

Fig. 2C and Fig. 2D tested mature DCs in isolation, using IFN γ to simulate signals from T cells. While the cytokine milieu in actual MLRs is more complex than this simplified system, the pattern observed in Fig. 2F (loss of inhibitory activity for T cells when DCs were matured) was consistent with the pattern shown in Fig. 2C (down-regulation of IDO when mature DCs
25 were activated with IFN γ). In contrast, the pattern shown in Fig. 2G (maintenance of inhibitory activity in MLR despite maturation) was consistent with Fig. 2D (expression of functional IDO in mature DCs). It is currently unknown what factors in the MLRs favored maintenance of IDO-mediated suppression in the large majority of experiments. One potentially significant variable could be the production of regulatory cytokines such as IL10 by the activated T cells. This issue
30 will require further investigation to elucidate. However, the key observation arising from Figs.

2D and 2G is that the CD123⁺ subset of DCs has the potential for IDO-mediated inhibition of T cells even when mature.

Donor-to-donor variability

5 Inhibition of T cell proliferation by immature DCs was seen in 10/10 experiments using seven different donors. In seven of these ten experiments, T cell proliferation was significantly enhanced by 1MT. With mature DCs, inhibition of T cell proliferation was observed in 12/13 experiments (six different donors) using sorted CD123⁺ DCs (BCS system) as shown in Fig. 2H. Also with mature DCs, inhibition was observed in 28/32 experiments (nine different donors)
10 using non-adherent IDO-enriched cells (SFM system) as shown in Fig. 2G. In four experiments using mature DCs (four donors), the DCs showed loss of inhibition upon maturation, the pattern shown in Fig. 2F. However, when two of these four donors were tested in multiple experiments it was found that by using different conditions (different maturation regimen, different responder T cells), these donors could be induced to show inhibition after maturation. Thus, the majority
15 of donors showed IDO-mediated inhibition by mature DCs, and this was subject to influence by the conditions prevailing during maturation and MLR.

Use and limitations of 1MT

20 In all experiments, the D-isomer of 1MT was used unless otherwise specified, because it gave better reversal of suppression with less toxicity than the DL- racemic mixture used previously (Munn et al., *Science*, 1998; 281:1191). As previously published (Munn et al., *J. Exp. Med.*, 1999;189:1363), 1MT is not an efficient inhibitor of IDO (K_m of approximately 30 uM). When the number of IDO⁺ DCs is large, a racemic mixture of 1MT is only partially effective in reversing IDO-mediated inhibition of T cell proliferation. While this could in theory imply a
25 second, unrelated mechanism of suppression that happened to co-segregate with IDO in our system, it more likely reflects the less-than-perfect efficiency of a racemic mixture of 1MT as an inhibitor of IDO. The effectiveness of 1MT was significantly enhanced if the DCs were matured (e.g., Fig. 2G), which may reflect a reduced potency of the IDO system in mature DCs (as suggested by Fig. 2C), and the greater inherent stimulatory capacity of mature DCs.

Expression of IDO protein without constitutive activity

Figs. 2B-2E suggest that IDO may exist in both enzymatically-active and inactive forms. This type of regulation, in which the functional enzymatic activity of a pre-formed pool of protein is turned on and off by post-translational events, is familiar in other enzyme systems.

5 Examples include regulation of protein kinases by phosphorylation in kinase cascades, regulation of signal-transduction proteins by farnesylation, and others. The shift between active and inactive states is required for the proper biologic function of these enzymes. It is not yet known whether there exist post-translational modifications that regulate IDO activity. However, it is known that factors which affect the heme prosthetic group of IDO can markedly alter enzymatic

10 activity (Thomas et al., *J. Biol. Chem.*, 1994;269:14457; Thomas et al., *J. Immunol.*, 2001;166: 6332) without necessarily affecting protein levels (Thomas et al., *J. Immunol.*, 2001;166:6332), so the possibility that IDO could exist in both active and inactive forms is not without precedent. Consistent with this, expression of immunoreactive IDO protein without functional enzymatic

15 activity has recently been described in subsets of murine DCs (Fallarino et al., *Int. Immunol.*, 2002;14:65). Fig. 3D shows data consistent with the possibility that activation of M ϕ s with IFN γ may induce post-translational modifications of IDO. This is suggested by the emergence of two additional protein species on 2D-gel electrophoresis after activation with IFN γ . These species react with the anti-IDO antibody on western blot (Fig. 3D) and migrate similarly to the major species in the SDS-PAGE dimension, but display shifted isoelectric points. Such shifts in pI

20 may be caused by alternate splicing, or, more commonly, may reflect post-translational modifications such as phosphorylation or acylation.

Thus, it appears that a constitutive presence in immature DCs of immunoreactive IDO protein, but with functional enzymatic activity being induced only after IFN γ activation, reflects an additional layer of specific, biologically relevant regulation of IDO. In this regard, it should

25 also be noted that, while flow cytometry failed to detect significant IDO in resting MCSF-derived M ϕ s (Fig. 2A), the more sensitive western blot analysis of detergent-solubilized whole-cell lysates reveals a pool of constitutive IDO protein even in resting M ϕ s (Figs. 3B and 3D), just as in immature DCs.

Example 2

A Small Population of Dendritic Cells in Tumor-draining Lymph Nodes Mediates Dominant Immunosuppression via Indoleamine 2,3-dioxygenase

5 The specific role of tolerogenic dendritic cells (DCs) in tumor immunology remains unclear. In part, this is because the specific molecular mechanisms by which DCs create tolerance are still poorly understood (Moser, *Immunity*, 2003;19:5). The present example focuses on the immunoregulatory enzyme indoleamine 2,3-dioxygenase (IDO) (Taylor et al., *FASEB J.*, 1991;5:2516). IDO is a tryptophan-degrading enzyme; its expression by cultured 10 macrophages and DC allows them to inhibit T cell proliferation in vitro (Munn et al., *J. Exp. Med.*, 1999;189:1363; Hwu et al., *J. Immunol.*, 2000;164:3596; and Munn et al., *Science*, 2002;297:1867). Transfection of recombinant IDO into tumor cell lines confers the ability to inhibit antigen-specific T cell responses in vitro (Mellor et al., *J. Immunol.*, 2002;168:3771), and protects immunogenic tumor from rejection in vivo (Uyttenhove et al., *Nat. Med.*, 2003;9:1269).

15 Endogenous IDO has been implicated in maternal tolerance toward the allogeneic fetus (Munn et al., *Science*, 1998;281:1191), tolerance to self antigens in NOD mice (Grohmann et al., *J. Exp. Med.*, 2003;198:153), and as a downstream effector mechanism for the tolerance-Inducing agent 20 CTLA4-Ig (Grohmann et al., *Nat. Immunol.*, 2002;3:985). Thus, IDO represents a potent endogenous immunoregulatory system that may be exploited by some tumors as a mechanism of immune evasion.

IDO can be expressed by a variety of human tumors and tumor cell lines (Uyttenhove et al., *Nat. Med.*, 2003;9:1269; Logan et al., *Immunol.*, 2002;105:478; and Friberg et al., *Int. J. Cancer*, 2002;101:151). This observation, combined with evidence from IDO-transfected tumors (Uyttenhove et al., *Nat. Med.*, 2003;9:1269), suggests that expression of IDO by 25 malignant cells may contribute to local immunosuppression within tumors. As shown in Example 1, IDO is expressed by a population of host cells found in certain tumor-draining lymph nodes (TDLNs), indicating that these cells represent a population of immunosuppressive host DCs that are recruited by the tumor. In the present example, a murine tumor model was developed to isolate and characterize the IDO-expressing DCs from TDLNs was developed and 30 used to study IDO-dependent T cell suppression.

The present example demonstrates that tumor-draining lymph nodes in mice contain a population of plasmacytoid dendritic cells (pDCs) that constitutively express immunosuppressive levels of the enzyme indoleamine 2,3-dioxygenase (IDO). These cells expressed multiple markers of mature pDCs, but also co-expressed CD19 and pax5, suggesting derivation from a lymphoid B-cell progenitor. CD19⁺ pDCs comprised less than 0.5% of cells in tumor-draining lymph nodes (TDLN) but could potently and dominantly suppress CD8⁺ T cell responses in vitro, in an IDO-dependent fashion. Adoptive transfer of DCs from tumor-draining lymph nodes created T cell unresponsiveness and dominant immunosuppression to a strong nominal antigen in naive, non-tumor-bearing hosts. These effects were abrogated by targeted disruption of the IDO gene in the transferred DCs, or by administration of the IDO inhibitor drug 1-methyl-tryptophan following adoptive transfer. These results indicate that IDO-expressing DCs create a local microenvironment in tumor-draining lymph nodes that suppresses host anti-tumor cytotoxic T cell responses.

15 *Materials and Methods*

Clinical studies. A series of samples from patients with malignant melanoma were randomly selected, based on the following inclusion criteria: radiographically mapped sentinel LN at the time of diagnosis; no metastases at presentation; and no further therapy given following initial surgical resection. Sentinel LN biopsies were stained for IDO by immunohistochemistry, expression was graded by three pathologists as described (Lee et al., *Lab. Invest.*, 2003;83:1457), and a consensus score obtained. Patients were stratified into normal (grade 0) versus abnormal (grade 1+ or higher) expression, and compared by Kaplan-Meier survival analysis. Sentinel LN biopsies from patients with breast cancer were selected from the archives of the Medical College of Georgia. Studies with human subjects were approved by the appropriate Institutional Review Board.

Mouse tumor models. C57BL/6 mice (Jackson, Bar Harbor, ME) were implanted with tumors in the anteriomedial thigh, using either 4×10^4 B16F10 (ATCC, Bethesda, MD) or 1×10^6 B78H1•GM-CSF. B78H1•GM-CSF cells recruit large numbers of APCs (Dranoff et al., *Proc. Natl. Acad. Sci. USA*, 1993;90:3539) and were originally designed as a vaccine strategy. GMCSF-transfected tumors are immunogenic if lethally irradiated prior to injection (Borrello et al., *Hum. Gene Ther.*, 1999;10:1983), but live tumors grow progressively and create systemic

tolerance to tumor antigens (Bronte et al., *J. Immunol.*, 1999;162:5728). Thus, B78H1•GM-CSF represented an excellent model for our studies, since it recruited many DCs but nevertheless provoke a protective immune response. Tumors were 10-12 days after implantation, well prior to the stage of large, metastatic tumors (day 21) in which the hosts began to show alteration in 5 spleen and non-tumor-draining LNs. FACS analysis showed that the pDCs recruited by B78H1•GM-CSF tumors were phenotypically identical to those recruited by B16F10 tumors, but with the advantage that they could be recovered in quantities sufficient for functional analysis. Immunohistochemical studies of established B16F10 and B78H1•GM-CSF tumors showed that neither expressed IDO in the tumor cells themselves. Studies, presented herein, using IDO- 10 deficient hosts formally showed that the relevant IDO expression was in host-derived APCs, not the tumor cell lines themselves.

15 Immunohistochemistry. Immunohistochemistry was performed on human materials as described in Example 1, following proteinase K antigen retrieval of formalin-fixed, paraffin-embedded sections. Mouse tissues were fixed overnight in 10% formalin and paraffin embedded. Since the IDO epitope was not stable to prolonged storage in thin sections, staining was performed within 24 hours of sectioning. Staining was performed using a rabbit anti-mouse IDO polyclonal antibody (Mellor et al., *J. Immunol.*, 2003;171:1652). Cytocentrifuge 20 preparations were made from cell suspensions following fluorescence-activated cell sorting, fixed for 10 minutes in 10% formalin, and stained within 24 hours. Controls for human and mouse staining included the anti-IDO antibody neutralized with a molar excess of the immunizing peptide.

25 Flow cytometry and cell sorting. Single-cell suspensions of LNs were obtained by teasing and disaggregation through a 40 micron mesh. Spleen cells were obtained by ground-glass homogenization and hypotonic lysis of erythrocytes. Cells were stained by 4-color immunofluorescence, using CD11c versus B220 versus CD19 versus a panel of other markers. Fc binding was blocked using a commercial anti-CD16/CD32 cocktail (BD Pharmingen, San Diego, CA). All acquisitions and sorts were performed using pulse-processing doublet discrimination. Antibodies against the following antigens were from BD-Pharmingen: CD11c (clone HL3), B220 (clone RA3-6B2), CD19 (clone 1D3), CD4 (clone H129.19), CD8 α (clone 30 53.6.7), Ly6c (clone AL-21), CD45RA (clone HI100), MHC class II (anti-I-A b , clone 25-9-17), CD80 (clone 16-10A1), CD86 (clone GL1), H-2K b (clone AF6-88.5), CD123 (clone 5B11), and

CD135/FLT3 (clone A2F10.1). Anti-mouse CCR6 (clone 140706) was from R&D Systems (Minneapolis, MN). Anti-clonotypic antibody Ti98 against the BM3 TCR was biotinylated and used as described (Tarazona et al., Int. Immunol., 1996;8:351). All antibodies were used with isotype-matched negative controls. To adjust for differences in nonspecific binding between cell 5 populations, each isotype control was gated on the specific population of interest. Analytical flow cytometry was also performed on a 4-color FACS-Caliber (Becton-Dickinson) with similar results.

T cell activation in MLR. BM3 responder T cells were prepared from spleen by nylon-wool enrichment. Stimulators (sorted DCs or unfractionated TDNL) were mixed with 1 x 10⁵ 10 BM3 responder cells in 200 ul IMDM at the ratios shown in each figure. After 3 days, proliferation was measured by 4-hour thymidine incorporation assay. Where indicated, replicate groups of wells received 200 uM 1-methyl-[D]-tryptophan (Sigma-Aldrich, St. Louis, MO) or 250 uM [L]-tryptophan (Sigma). To prepare a 100x 1MT stock, a 20 mM solution was dissolved 15 in 0.1 N NaOH and then adjusted to pH 7.4. All MLRs were performed in V-bottom culture wells (Nalge-Nunc, Rochester, NY), as previously described in Example 1, because the close cell-cell contact gave the maximum sensitivity to IDO-mediated suppression.

In these studies, it was important that the stimulator DCs not be irradiated, because initial validation studies showed that irradiation significantly altered the viability and functional attributes of IDO⁺ pDCs. MLRs were thus “two-way” reactions. However, the small number of 20 sorted DCs used as stimulators contributed negligible proliferation compared to the large population of TCR-transgenic responder cells. Since the relevant readout was dominant suppression, the two-way MLR design presented no problem in interpretation.

Quantitative real-time PCR. Total RNA was extracted from sorted cells using Trizol 25 (Gibco-BRL, Gaithersburg, MD). RNA was reverse-transcribed and amplified using the LightCycler real-time PCR system (Roche, Pleasanton, CA) with the RNA SYBR Green kit (Roche). All groups were compared in the same run, and quantitated against a standard curve of spleen RNA. Primers for mouse γ -actin were as follows. Sense was GATGACGCCAGATAATGTTT (SEQ ID NO:2) and antisense was TCTCCTTATGTCACGAAC (SEQ ID NO:3), yielding a 290 basepair product. Primers for 30 mouse CD19 were as follows. Sense was GGCACCTATTATTGTCTCCG (SEQ ID NO:4) and antisense was GGGTCAGTCATTCGCTTC (SEQ ID NO:5), yielding a 218 basepair product).

The primers for mouse pax5 were as follows. Sense was GCATAGTGTCTACAGGCTCCG (SEQ ID NO:6) and antisense was GATGGGTTCCGTGGTGGT (SEQ ID NO:7), yielding a 299 basepair product. Conditions were optimized for each set of primers to give a linear standard curve over a 1000-fold range, with a correlation coefficient of r greater than 0.99. An 5 aliquot of each experimental sample was pre-screened to determine γ -actin message, then each sample was loaded to amplify an equivalent amount of γ -actin. For gels, RT-PCR was performed for the pre-determined optimum number of cycles yielding amplification in the linear range, and products resolved by formaldehyde gel electrophoresis. All primers gave single bands of the expected molecular weight.

10 Adoptive transfer studies. Recipient CBA mice (Jackson) were prepared by intravenous injection of 4×10^7 BM3 splenocytes (termed CBA+BM3 mice). It has been previously shown that adoptively transferred BM3 T cells are stable in CBA hosts for over 100 days (Tarazona et al., *Int. Immunol.*, 1996;8:351). CD11c $^+$ DCs were isolated from TDLNs, or from normal LNs of C57BL/6 and CBA mice, using a Cytomation MoFlo high-speed cell sorter. Two aliquots of 15 1×10^5 DCs were injected into each recipient, subcutaneously in each anteriomedial thigh (analogous to the position of the original tumor). After 10 to 12 days, recipient mice were euthanized and the inguinal LNs (draining the sites of injection) and spleen (a representative distant site) removed for analysis. In some experiments, mice received 1-methyl-[D]-tryptophan (compound designation NSC 721782, Drug Development Group, Division of Cancer Treatment 20 and Diagnosis, National Cancer Institute, Rockville, MD) by continuous subcutaneous infusion (5 mg/day) using implantable copolymer pellets as previously described (Munn et al., *Science*, 1998; 281:1191). Control mice received vehicle pellets alone.

IDO-expressing cells in human TDLNs predict a poor clinical outcome

25 Screening studies of radiographically mapped tumor-draining (sentinel) lymph nodes from patients with breast carcinoma and malignant melanoma demonstrated that a subset of patients had abnormal accumulation of cells expressing IDO in sentinel nodes. Similar cells were also seen in regional LNs dissections from other solid tumors (colon, lung and pancreas, not shown). Melanoma was chosen for further analysis because of the availability of archived 30 sentinel LNs with paired long-term clinical follow-up. In a retrospective study of 40 patients with malignant melanoma, the presence of an abnormal number of IDO $^+$ cells in the sentinel LN

at the time of diagnosis was found to be a significantly adverse prognostic factor. None of these patients had detectable metastases at the time of the biopsy, so recruitment of IDO⁺ cells occurred early in the course of the disease. In this example, a murine system to model these cells was developed and used to determine that the IDO-expressing cells in TDLNs represented a 5 population of immunoregulatory APCs.

IDO-expressing cells in murine TDLNs

The presence of IDO⁺ cells in TDLNs of mice with Lewis Lung Carcinoma has been previously reported (Friberg et al., *Int. J. Cancer*, 2002;101:151). With the present example, 10 IDO⁺ cells were also found in TDLNs in the well-characterized B16F10 melanoma model. LNs were harvested 7-12 days after tumor implantation, well before any detectable metastases to the LNs. The accumulation of IDO⁺ cells occurred only in LNs draining the tumors; few or none were present in contralateral (non-tumor-draining) LNs from the same animals.

Morphologically, the IDO⁺ cells were plasmacytoid mononuclear cells similar to those found in 15 human nodes. However, the number of such cells recruited by B16F10 tumors was lower than observed in heavily-infiltrated human LNs. To increase the number of APCs in the draining LNs, a subline of B16 melanoma (clone B78H1) transfected with the cytokine GM-CSF (Huang et al., *Science*, 1994;264:961) was also stained for IDO expression. GM-CSF markedly increases the number of APCs recruited into TDLNs (Dranoff et al., *Proc. Natl. Acad. Sci. USA*, 20 1993;90:3539), and many primary human melanomas and other tumors constitutively express mRNA for GM-CSF (Mattei et al., *Int. J. Cancer*, 1994;56:853; Smith et al., *Clin. Exp. Metastasis*, 1998;16:655; Colasante et al., *Hum. Pathol.*, 1995;26:866; and Bronte et al., *J. Immunol.*, 1999;162:5728). B78H1•GM-CSF tumors recruited large number of IDO-expressing cells, comparable to heavily infiltrated humans LNs. As in B16F10, IDO expression was 25 localized to the draining node, and was not observed in contralateral nodes from the same mice, nor in systemic sites such as spleen. Subsequent experiments therefore used TDLNs from mice with B78H1•GM-CSF tumors

TDLNs contain a population of suppressive plasmacytoid DCs

30 Single-cell suspensions of TDLNs, or the paired contralateral LNs, were used as stimulator cells in mixed leukocyte reactions (MLRs). The responder cells in MLRs were TCR-

transgenic CD8⁺ T cells from BM3 mice, which recognize a nominal antigen (H-2K^b) constitutively expressed by all APCs from the C57BL/6 tumor-bearing hosts (Tarazona et al., *Int. Immunol.*, 1996;8:351). Preliminary validation studies confirmed that expression of the target antigen was high and comparable in DCs from TDLNs and normal LNs. Fig. 5A shows that 5 cells from TDLNs were poor stimulators of BM3 T cells, whereas cells from the contralateral (non-tumor-draining) LNs of the same animals were excellent stimulators (comparable to LN cells from non-tumor-bearing mice, not shown). Mixing experiments (Fig. 5A, right panel) revealed that the failure of responder cells to proliferate in response to TDLN cells was due to a dominant suppressor activity present in the TDLN cells.

10 Sorting experiments (Fig. 5B) revealed that a suppressor activity was present in a CD25⁺CD4⁺ fraction (2-3% of total cells) corresponding to the Treg population known to be present in mice with B16 tumors (Sutmuller et al., *J. Exp. Med.*, 2001;194:823). Further sorting revealed that a second, equally potent suppressor activity was also present in the CD11c⁺B220⁺ fraction (1-2% of total cells) comprising plasmacytoid DCs (pDCs). When these two suppressor 15 populations were removed, the remaining 95-97% of TDLN cells stimulated excellent proliferation (as expected, since this fraction included all DCs other than pDCs, plus all B cells). Mixing experiments showed that suppression by pDCs was dominant, and was also quite potent, since the 1-2% of pDCs was able to suppress responses stimulated by all of the other APCs.

20 *Immunosuppression by pDCs is mediated by IDO*

To test whether T cell suppression by pDCs was mediated by IDO, MLRs were performed in the presence or absence of the IDO inhibitor 1-methyl-tryptophan (1MT). Fig. 5C shows that 1MT blocked the suppressive activity of pDCs, converting them into effective 25 stimulators of T cell proliferation, and reversing their dominant suppression in mixing experiments. This was not due to any nonspecific activating effects of 1MT on T cells, because the same T cells stimulated by the non-suppressive “all other” fraction showed no enhancement by 1MT.

To further confirm that suppression by pDCs was mediated via IDO, tumors were implanted in mice with a targeted disruption of the IDO gene (IDO-knockout mice). The pDCs 30 isolated from TDLNs in these mice showed no suppressor activity (Fig. 5C, right panel). Further

confirming the specificity of 1MT for IDO, 1MT had no effect on MLRs stimulated by IDO-deficient pDCs.

Finally, the suppressive effect of pDCs was abrogated by adding supraphysiologic, ten fold increased (10x), levels of L-tryptophan to the MLR (Fig. 5D). This prevents IDO from 5 depleting tryptophan, via simple substrate excess, rather than by enzyme inhibition as with 1MT, and circumvents IDO-mediated suppression of T cells (Munn et al., *J. Exp. Med.*, 1999;189:1363). Excess L-tryptophan abrogated suppressor activity in a fashion similar to 1MT. Also as with 1MT, there was no effect of 10x L-tryptophan when pDCs were derived from IDO-KO hosts (Fig. 5D, right panel).

10

Adoptive transfer of DCs from TDLNs creates immunologic unresponsiveness in vivo

Next, whether the DCs from TDLNs were sufficient to create immunologic unresponsiveness in vivo, independent of the original tumor, was addressed. CD11c⁺ DCs were isolated from TDLNs and adoptively transferred to new hosts. For these experiments, all of the 15 DCs from TDLNs were used, not just the pDCs, in order to accurately reflect the mixed population found in TDLNs, and to ask whether stimulation of suppression would predominate in vivo. Ten days after adoptive transfer, T cells in the recipient host were tested for responsiveness to a nominal antigen (H-2K^b) presented by the transferred DCs. Recipients were allogeneic (H-2K^b-negative) CBA mice that had been pre-loaded with a large cohort of H-2K^b- 20 specific BM3 T cells.

Fig. 6A shows that adoptively transferred DCs induced selective accumulation of antigen-specific BM3 T cells in inguinal LNs of recipient mice. This accumulation was comparable whether DCs were derived from TDLNs or from normal C57BL/6 LNs (both of which expressed H-2K^b). In contrast, DCs from antigen-negative CBA mice caused no such 25 accumulation. In all groups, the number of BM3 T cells in the spleens (a site where BM3 T cells passively accumulate after injection) was similar, indicating that the initial loading was comparable.

Inguinal LN cells from recipient mice were assayed for functional responsiveness using MLRs stimulated by irradiated C57BL/6 (H-2K^b-positive) splenocytes. Fig. 6B shows that T 30 cells from mice receiving TDLN DCs displayed profound hyporesponsiveness to recall antigen stimulation, despite the presence of ample BM3 T cells (see, Fig. 6A). In contrast, T cells from

control animals receiving normal C57BL/6 DCs (containing a comparable number of BM3 T cells) displayed a brisk MLR response. Mice receiving antigen-negative DCs also responded well in MLRs, confirming that the large cohort of pre-positioned transgenic BM3 cells allowed a vigorous ex vivo response without the need for any previous priming.

5 To test whether the state of acquired T cell unresponsiveness was created by IDO expression in the transferred TDLN DCs, recipient mice were treated with the IDO inhibitor 1MT at the time of adoptive transfer (control mice received pellets without 1MT). After 10 days, T cells were harvested and tested for responsiveness to antigen. Recall MLRs did not contain 1MT. Fig. 6C shows that administration of 1MT prevented the induction of T cell 10 unresponsiveness in the recipients. The effect was specifically to block the acquisition of unresponsiveness, not simply to enhance T cell responses in general, since 1MT had no enhancing effect on mice receiving normal DCs (right-hand panels). In all experiments, flow cytometry confirmed that LNs from all groups contained comparable numbers of BM3 T cells, similar to Fig. 6A.

15 In the same experiments, the systemic response of T cells isolated from spleens were also tested (Fig. 6C, lower panels). T cells from this remote site showed a modest reduction in response compared to controls, which was prevented by 1MT treatment, but T cells in the spleen retained significantly more responsiveness than T cells from draining LNs. Thus, unresponsiveness was most profound in the LNs directly draining the site of injection.

20

T cells in spleen become aware of antigen introduced on IDO⁺ DCs

It was not clear whether T cells in the spleen became “aware” of antigens introduced on the transferred DCs. To address this question, the down regulation of clonotype-specific TCR on transgenic T cells was examined as a surrogate marker for antigen encounter (Tafuri et al., 25 *Science*, 1995;270:630). Although descriptive, this helps make the important distinction between true immunologic “ignorance” (no encounter with antigen) from encounter without activation. In vitro, it was found that BM3 T cells encountering antigen on TDLN cells showed a rapid and sustained down regulation of clonotype-specific TCR (Fig. 7A); in contrast, control BM3, stimulated by cells from normal LNs, retained TCR expression. Thus, sustained down regulation 30 of TCR served as a marker for encounter with TDLN cells.

Applying this marker to adoptive transfer studies (Fig. 7A), it was found that BM3 T cells from hosts receiving TDNL DCs showed a uniform pattern of clonotype-specific TCR down regulation. In contrast, hosts receiving normal DCs showed no down regulation. This was observed in the LNs draining the site of adoptive transfer, but was equally present in spleens from the same animals. Thus, even at distant sites such as spleen, responding T cells were not truly “ignorant” of antigen, but had been affected the adoptively-transferred DCs (even though the affected cells were not anergic); and this effect was specific for TDNL DCs compared to normal DCs. When TDNL DCs were derived from IDO-knockout mice, there was little TCR down regulation following adoptive transfer (Fig. 7B), suggesting that most of the down regulation was mechanistically due to IDO expression by the TDNL DCs. A similar effect was seen when 1MT was administered following adoptive transfer, also supporting the role of IDO. IDO-deficient TDNL DCs also did not create functional T cell unresponsiveness in the recipients (Fig. 7B, right panel), similar to recipient mice treated with 1MT (Fig. 6C).

15 *IDO⁺ DCs create secondary suppressor mechanisms that are independent of IDO*

Next, it was addressed whether the T cell unresponsiveness created by adoptive transfer was due to intrinsic anergy of the responding T cells, or to some form of active suppression. Mixing experiments were performed using two groups of responder cells. One group of responder cells was control T cells isolated from CBA+BM3 recipients following adoptive transfer of normal DCs (fully responsive); and the second group of responder cells was T cells from the same mice following adoptive transfer of TDNL DCs (unresponsive). Mixing experiments (Fig. 7C) showed that the “unresponsive” cells entirely suppressed proliferation by the otherwise competent population (Fig. 7C, arrow). This was thus not consistent with simple anergy on the part of the unresponsive cells, but instead indicated a component of active, dominant suppression.

20 To test whether this suppression was mediated by IDO, recall MLRs were performed in the presence or absence of 1MT. Fig. 7C (right panel) shows that 1MT had no effect on suppression in recall MLRs, implying that IDO was not the mechanism of suppression. Thus, while the creation of unresponsiveness was absolutely dependent on IDO during its induction phase (Figs. 6C and 7B), the unresponsive state was maintained, at least in part, by additional, IDO-independent mechanisms.

IDO-mediated suppression segregates with a small subset of CD19⁺ DCs

The preceding studies showed that some IDO-expressing cell type in TDLNs was responsible for creating immunologic unresponsiveness in vivo. To better define the specific DC subset, more detailed sorting experiments were performed. The experiments in Fig. 5 had shown that IDO-mediated suppressor activity segregated with the plasmacytoid (B220⁺) DC fraction. Further phenotyping studies of this population showed it to be heterogeneous with respect to a number of markers, in particular expression of CD19. DCs from TDLN cells were sorted into CD19⁺ and CD19^{NEG} fractions of pDCs, as well as conventional B220^{NEG} DCs, following the schema shown in Fig. 8. Immunohistochemistry on the sorted cells revealed that all three populations contained at least some cells with immunoreactive IDO. To quantitatively measure the IDO-mediated suppressor activity associated with each subset, the fractions were analyzed as stimulators in MLRs with and without 1MT. Control MLRs received B cells from the same sorting run, which were good stimulators with no suppressor activity. Fig. 8 shows that virtually all of the IDO-mediated suppressor activity segregated with the novel CD19⁺ DC fraction. When these CD19⁺ DCs were removed, the remaining DCs showed minimal suppression (Figs. 8B and 8C).

Despite comprising almost all of the IDO-mediated suppression, CD19⁺ pDCs were a small fraction of total LN cells. In a total of 13 experiments (analyzing 2-6 pooled TDLNs each), the total CD11c⁺ DCs were found to comprise 1-1.5% of cells. Of these, CD19⁺ pDCs comprised 31±15% of DCs. Thus, the potent IDO-induced suppression seen in Fig. 5B, capable of dominantly inhibiting the proliferation stimulated by all of the other nonsuppressive DCs and B cells in mixing experiments, was mediated by 0.3-0.5% of total LN cells.

CD19⁺ DCs show a phenotype of mature plasmacytoid DCs

Fig. 9A shows immunophenotyping of cells from TDLNs, gated first on the CD11c⁺ DCs, then further gated into CD19⁺ and CD19^{NEG} fraction. Other markers (including B220) were analyzed in the third and fourth colors. Fig. 9A shows that all of the CD19⁺ DCs in TDLNs expressed B220, consistent with their being a subset of pDCs (although not all of the B220⁺ pDCs expressed CD19). Many of the CD19⁺ DCs also expressed CD4 and/or CD8a, both of which can be found on pDCs (and both of which served to unambiguously distinguish CD19⁺

pDCs from B cells). Many of the CD19⁺ DCs also expressed Ly6c and CD45RA, which are markers associated with murine plasmacytoid DCs (O'Keeffe et al., *J. Exp. Med.*, 2002;196:1307; and Martin et al., *Blood*, 2002;100:383). CD19⁺ DCs also uniformly expressed the receptor tyrosine kinase Flt3 (CD135), which is expressed on DCs but not on mature B cells (Karsunky et al., *J. Exp. Med.*, 2003;198:305).

To confirm that the apparent expression of CD19 was authentic, mRNA for CD19 was measured by quantitative RT-PCR. The upstream transcription factor pax5 was also measured, since expression of CD19 is obligately dependent on expression of pax5 (Nutt et al., *EMBO J.*, 1998;17:2319; and Mikkola et al., *Science*, 2002;297:110). Fig. 9B confirms that cells of the CD19⁺ pDC fraction expressed mRNA for both CD19 and pax5. Quantitatively, these genes were present at somewhat lower levels than in mature B cells (normalized to γ -actin), but they were expressed at much higher levels than in either the CD19^{NEG} fraction of pDCs, or the B220^{NEG} conventional DCs, and were thus unambiguously positive.

In other experiments CD19⁺ pDCs of the same phenotype (CD11c⁺ CD19⁺, B220⁺, Ly6c⁺, CD45RA⁺) were also found in normal LNs from non-tumor-bearing hosts, as well as in the contralateral LNs of mice with B78H1•GM-CSF tumors, and in TDLNs from mice with B16F10 tumors. In the normal LNs they comprised 18±6% of total DCs (n=6 experiments), compared to 31±15% of total DCs in B78H1•GM-CSF TDLNs.

CD19⁺ pDCs TDLNs uniformly expressed high levels of MHC class II molecules, and costimulatory molecules CD80 and CD86, suggestive of a mature phenotype (Fig. 9C). These were expressed at levels equal to or greater than the CD19^{NEG} DCs from the same LN, many of which were immature, as shown. In different TDLNs, the CD19^{NEG} subset of DCs showed significant variability in the number of mature versus immature DCs; however, all of the CD19⁺ pDCs were invariably mature.

In addition to maturation markers, the CD19⁺ pDCs also expressed two markers observed in the human system. As shown in Example1, CD123 (IL3Ra) and the chemokine receptor CCR6 segregate closely with IDO expression in human monocyte-derived DCs and macrophages. In this example, it was found that in murine TDLNs, expression of CD123 and CCR6 preferentially associated with the CD19⁺ subset of DCs in TDLNs (Fig. 9D). In contrast, the majority of CD19^{NEG} DCs were low or negative for these markers (Fig. 9D), as were the

majority of B cells, which were less than 20% positive for CCR6 and less than 2% positive for CD123.

Discussion

5 The current example identifies a small population of immunoregulatory DCs in TDLNs that are capable of mediating dominant immunosuppression in vitro, and creating profound immunologic unresponsiveness in vivo. Of the limited number of studies assessing DCs from TDLNs, most have reported a defect in their ability to stimulate T cells (Vicari et al., *J. Exp. Med.*, 2002;196:541; Almand et al., *Clin. Cancer Res.*, 2000;6:1755; and Yang et al., *J. Clin. Invest.*, 2003;111:727). However, it has been unclear whether this was due to immaturity of the DCs, or to some form of active suppression. This example shows that one subset of DCs in TDLNs can create potent active suppression, mediated via the molecular mechanism of IDO.

10 The suppression created by IDO-expressing DCs was profound, but it was fundamentally local. First, the expression of IDO itself was strictly localized, being found in the LNs draining tumors, but not in other LNs from the same animal. Second, adoptive transfer of DCs from these 15 nodes created complete T cell unresponsiveness in the new nodes draining the site of injection, while T cells in spleens of the same animals remained reactive. An analogous dichotomy has been observed in tumor-bearing hosts, where tumors may be locally tolerated despite the 20 systemic presence of competent, tumor-specific T cells (Wick et al., *J. Exp. Med.*, 1997;186:229; Speiser et al., *J. Exp. Med.*, 1997;186:645; and Nguyen et al., *J. Exp. Med.*, 2002;195:423). The fact that tumors in this situation grow unchecked, ultimately killing their hosts, emphasizes the point that immunosuppression does not have to be systemic in order to be effective. Functionally, if all the draining LNs of all the sites of tumor create local unresponsiveness, the tumor is de facto tolerated.

25 In this regard, it is relevant to note that the immunosuppression created by IDO-expressing DCs was dominant. This was shown by in vitro mixing experiments, in which a small population of CD19⁺ pDCs from TDLNs could completely suppress T cell responses, despite the presence of many other stimulatory APCs (for example, see Fig. 5). It remains to be elucidated how such a small population of IDO⁺ DCs can effectively control a large T cell 30 response, but this same phenomenon has been observed in other studies of IDO-expressing DCs (Grohmann et al., *J. Immunol.*, 2000;165:1357; and Mellor et al., *J. Immunol.*, 2003;171:1652).

This example demonstrated that the adoptive transfer of TDNL DCs *in vivo* was able to create local immunologic unresponsiveness to a strong nominal antigen (H-2K^b), even in the presence of a large cohort of antigen-specific, TCR-transgenic T cells. This local effect was accompanied by a systemic effect on all transgenic T cells, as shown by sustained down regulation of TCR expression. It has been suggested that such TCR down regulation may serve as a marker for anergy (Benson et al., *J. Clin. Invest.*, 2000;106:1031; and Tafuri et al., *Science*, 1995;270:630), but anergy alone could not account for all of the immunosuppression created by the TDNL DCs. Mixing experiments revealed that there was also a potent component of active suppression, capable of inhibiting other, fully competent responder T cells *in vitro* (see Fig. 7C).

5 This secondary suppressor activity was not itself mediated by IDO, but it was obligately dependent on expression of functional IDO by TDNL DCs for its creation. Thus, the IDO expressed by TDNL DCs elicited a secondary mechanism to amplify and sustain its immunosuppressive effect.

10 These observations are consistent with findings in other studies using CD8a⁺ DCs from spleen, activated *in vitro* to express IDO. Adoptive transfer of even small numbers of such IDO-expressing DCs was capable of creating systemic unresponsiveness to antigen (Grohmann et al., *J. Immunol.*, 2000;165:1357; and Fallarino et al., *Nat. Immunol.*, 2003;4:1206-12).

15 In the present system, the immunosuppression mediated by IDO-expressing DCs was an active process, not merely a passive failure of immature DCs to stimulate T cells. It is well established that immature DCs can be tolerogenic (Hawiger et al., *J. Exp. Med.*, 2001;194:769; and Probst et al., *Immunity*, 2003;18:713), which has been attributed to their failure to provide adequate costimulation. However, it has been more controversial whether certain DCs may be tolerogenic even when mature (Moser, *Immunity*, 2003;19:5). The CD19⁺ pDCs found in TDNLs appeared phenotypically mature (positive for CD80 and CD86, high MHC-II expression, and excellent stimulators of T cell proliferation when IDO was blocked). Despite this, they were also actively suppressive *in vitro*, as shown by mixing experiments. In order to clearly demonstrate the active nature of this suppression *in vivo*, the present adoptive transfer studies were designed so that vigorous T cell activation was the default response. A strong alloantigen (H-2K^b) was employed, constitutively expressed on all transferred DCs, recognized by a massive population of pre-positioned TCR-transgenic T cells (up to 40% of recipient CD8⁺ T cells). Because of this large “pre-expanded” clone of T cells, there was no need for any initial priming

step in order to see a robust proliferative response in MLR assays. Even in this system, DCs from TDLNs were able to create acquired unresponsiveness to the H-2K^b antigen, in an IDO-dependent fashion.

Fractionation studies of TDLN cells showed that virtually all of the IDO-mediated 5 suppressor activity segregated with a novel population of CD19⁺ DCs. CD19, and its obligate transcription factor pax5, are markers of the B cell lineage (Fearon and Carroll, *Ann. Rev. Immunol.*, 2000;18:393; and Nutt et al., *Nature*, 1999;401:556), so expression of these genes suggests derivation from a B-lineage precursor (or a common lymphoid progenitor cell). Consistent with this possibility, it is known that early CD19⁺ pro-B cells can give rise to DCs in 10 vitro (Bjorck and Kincade, *J. Immunol.*, 1998;161:5795; and Izon et al., *J. Immunol.*, 2001;167:1387). Recent analyses of both human and murine plasmacytoid DCs suggests a B-cell origin for a subset of pDCs (Rissoan et al., *Blood*, 2002;100:3295; and Corcoran et al., *J. Immunol.*, 2003;170:4926); in mice, up to one-third of DNA from murine splenic plasmacytoid DCs was found to show D-J rearrangement of the IgH locus (Corcoran et al., *J. Immunol.*, 15 2003;170:4926). Despite this link, previous studies have failed to identify the CD19⁺ subset of pDCs. In part, this may be because many studies have specifically excluded the CD19⁺ cells, either by depletion or by back-gating (O'Keeffe et al., *J. Exp. Med.*, 2002;196:1307; and Asselin-Paturel et al., *Nat. Immunol.*, 2001;2:1144). Even if recognized as DCs, it would not be obvious that the CD19⁺ cells were tolerogenic, because resting CD19⁺ DCs do not constitutively express 20 IDO, and are not suppressive. It was only in the context of TDLNs that the regulatory attributes of these cells became evident.

The key difference between TDLNs and normal LNs was not the presence or absence of 25 CD19⁺ DCs, as they were present in both, although at higher numbers in the TDLNs, but rather the fact that CD19⁺ DCs from TDLNs constitutively expressed IDO. It has been previously shown that the IDO-inducing agent CTLA4-Ig up regulates IDO preferentially in the B220⁺ and CD8a⁺ DC subsets (Mellor et al., *J. Immunol.*, 2003;171:1652). Prior to CTLA4-Ig treatment, these DCs were not inhibitory, but they became so after IDO was induced (Mellor et al., *J. Immunol.*, 2003;171:1652), indicating that some factor in TDLNs acts to induce constitutive expression of IDO in CD19⁺ pDCs. This factor might be a microenvironmental signal, such as a 30 cytokine, found selectively in TDLNs. Alternatively, it has recently been shown that CD25⁺CD4⁺ Tregs can induce IDO in DCs via expression of CTLA4 (Fallarino et al., *Nat.*

Immunol., 2003;4:1206-12). Thus, it is possible that TDLNs might contain a population of Tregs that triggers IDO. Elucidating the upstream factors responsible for the constitutive induction of IDO in TDLNs may offer significant insight into how tumors evolve to exploit the IDO mechanism.

5

Example 3

1-Methyl-[D]-Tryptophan, a Novel, Small-Molecule, Orally-Bioavailable Immune Modulator For Use in Cancer Immunotherapy

10 Tumors actively create a state of tolerance toward their own antigens. This pathologic situation allows the tumors to escape from host immune surveillance and also imposes a barrier to effective anti-tumor immunotherapy. One molecular mechanism by which tumors may inhibit immune responses is via the immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO). IDO degrades the amino acid tryptophan, and this acts to inhibit T cell responses. The small-
15 molecule 1-methyl-D-tryptophan (1MT) acts as an inhibitor of IDO enzyme activity in vitro, and is capable of preventing IDO-mediated immunosuppression in vivo. 1MT thus acts as an immune-enhancing agent in a variety of animal models where IDO limits or suppresses immunologic responses. In this example, administration of 1MT to tumor-bearing hosts in conjunction with low-dose chemotherapy or radiation shows synergistic immune-mediated anti-
20 tumor effect. 1MT thus represents a novel small-molecule, orally-bioavailable immune modulator for use in cancer immunotherapy.

25 1MT targets a previously unrecognized immunosuppressive pathway. This endogenous pathway may limit the efficacy of current immunotherapy approaches. One application of 1MT is as a vaccine adjuvant, since anti-tumor vaccines have shown occasional encouraging responses but their overall response rates remain limited (Yu and Restifo, *Journal of Clinical Investigation* 2002;110:289-294). Thus, adding 1MT as an adjuvant to an existing (Phase II) anti-tumor vaccine would be ethical and appropriate. Further, it is likely unnecessary to supply exogenous tumor antigen in the form of a vaccine. Chemotherapy and radiation already release large amounts of antigen from dying tumor cells, but these do not normally generate a useful immune response. 1MT will allow immune responses to such antigens.

Comparison of D- versus L- isomers of 1MT

Cultured human dendritic cells (DCs) enriched for IDO expression were prepared as described by Munn et al., *Science* 2002;297:1867-1870. DCs were used as stimulators in allogeneic mixed-leukocyte reactions (MLRs), with allogeneic lymphocytes as responder cells.

5 The ability of 1MT to inhibit IDO-mediated suppression is measured as the amount of T cell proliferation. Fig. 10 shows that the D isomer was significantly more effective than the L isomer at reversing IDO-mediated suppression.

IDO-expressing cells in human tumor-draining lymph nodes

10 Using a polyclonal antibody against human IDO, abnormal accumulation of IDO⁺ cells was seen in many tumor-draining (sentinel) lymph nodes from patients with malignant melanoma (Example 2 and as described in Lee et al., *Laboratory Investigation* 2003;83:1457-1466). A retrospective analysis of sentinel nodes from 40 patients with locally-confined disease at diagnosis showed the presence of these IDO⁺ cells was an adverse prognostic factor, even 15 prior to detectable metastasis.

IDO⁺ cells are selectively recruited into mouse tumor-draining lymph nodes

Tumor-draining lymph nodes from C57BL/6 mice implanted with B16F10 melanoma cells (day 14) showed significant accumulation of IDO⁺ cells compared contralateral lymph 20 nodes from the same animals. Phenotypically, the IDO⁺ cells were mostly CD11c⁺B220⁺ "plasmacytoid" dendritic cells. To obtain larger lymph nodes for functional studies, B78H1.GMCSF, a GMCSF-transfected sub-line of B16 was also used (Huang et al., *Science* 1994;264:961-965; and Borrello et al., *Human Gene Therapy* 1999;10:1983-1991). These tumors recruited the same IDO⁺ cells, but yielded quantitatively more cells for study. GMCSF- 25 transfected cell lines recruit large numbers of DCs (Dranoff, *Immunological Reviews* 2002;188:147-154), but as viable tumors they have been found to induce tolerance instead of spontaneous immunity (Bronte et al., *J. Immunol.* 1999;162:5728-5737).

IDO-mediated suppression segregates with the plasmacytoid DCs

30 Fig. 5 shows IDO-expressing suppressive APCs are present in tumor-draining LNs. Pooled draining LN cells (4 nodes) were stained and fractionated by 4-color MoFlo cell sorting

for the two populations shown in the schematic of Fig. 5B. All other cells were collected in a third fraction. Each fraction was then used as stimulators in MLRs, using 50,000 BM3 T cells as responders. The number of stimulators used in each MLR was adjusted to be the same as would have been present in 50,000 cells of the original LN preparation, based on the measured
5 percentage of each sorted fraction. Thus, 500 cells of the sorted B220⁺ CD11c⁺ fraction were added per well, and 1500 cells of the CD25⁺ CD4⁺ fraction, while 48,000 cells of the "all other" fraction were used. Replicate MLRs were performed with or without 1MT, as shown. Tumor-draining LN cells from a wild-type (IDO-sufficient) C57BL/6 host and tumor-draining LN cells from an IDO-knockout host, showing no IDO-mediated inhibition (but with inhibition by Tregs intact) are shown in Figs. 5C and 5D. The arrows in Fig. 5C indicate the IDO-mediated (1MT-sensitive) component of inhibition.
10

Cells were harvested from tumor-draining lymph nodes (day 12) and used as APCs in allogeneic mixed-leukocyte reactions (MLRs). Responder T cells were taken from "BM3" TCR-transgenic mice (CD8⁺, recognizing H-2K^b (Tarazona et al., *International Immunology* 15 1996;8:351-358)). Cells were sorted into B220⁺ CD11c⁺ plasmacytoid DCs, and a separate CD25⁺CD4⁺ fraction of regulatory T cells. It was important to separate out these Tregs, otherwise they would nonspecifically inhibit proliferation in the readout MLRs. The third fraction collected comprised the remaining 96% of the lymph-node cells; these cells stimulated good T cell proliferation, and there was no enhancement by 1MT (thus confirming that 1MT had 20 no nonspecific stimulatory effect in the absence of IDO). The plasmacytoid DC fraction was potently inhibitory, and this was fully reversed by 1MT. Mice with a targeted deletion of IDO were as described in Mellor et al., *J. Immunol.* 2003;171:1652-1655 9. These IDO-knockout mice showed no suppressor activity by plasmacytoid DCs, and no effect of 1MT, thus confirming that the molecular target of 1MT was indeed IDO.
25

Effect of 1MT on established tumors, and synergy with radiation or cyclophosphamide

B16F10 melanomas were implanted in C57BL/6 mice, then 7 days later mice were treated with 1MT or vehicle control (administered by SQ continuous infusion using implantable co-polymer pellets (Munn et al., *Science* 1998;281:1191-1193)). The initial studies used the DL 30 racemic form of 1MT, at a total dose of 20 mg per mouse per day. In this established-tumor model, 1MT alone had no effect. Not unexpected, given that the tumor had already been allowed

to create tolerance in the host. However, when the established host/tumor milieus was transiently perturbed by a single dose of total-body radiation (500 cGy) or cyclophosphamide (150 mg/kg x 1 dose), 1MT acted synergistically with both interventions, to significantly reduce tumor growth (Fig. 11A and 11B). Although the combination was not curative, this degree of growth delay

5 was comparable to that seen with other immunologic interventions in this aggressive tumor model (van Elsas et al., *J Exp Med* 1999;190(3):355-66; and Kotera et al., *Cancer Research* 2001;61(22):8105-8109). Identical experiments performed in immunodeficient (RAG1-knockout) hosts showed no enhancing effect of 1MT over cyclophosphamide alone (Fig. 11C), indicating that the effect of 1MT was entirely immunologically mediated. Finally, the [D]-10 isomer of 1MT was found to be effective at one quarter the dose used for the racemic preparation (Fig. 11D).

15 The radiation studies shown in Fig. 11A were replicated using a similar experimental design. The pattern of response observed in these studies was similar to that shown in Fig. 11A, with 1MT alone having no effect, but with the combination of the [D]-isomer of 1MT and radiation showing enhanced effect over radiation alone.

Pharmacokinetics

20 Comparative pharmacokinetic studies were initiated to characterize the in vivo disposition of the stereoisomers of 1-MT when administered by different routes, to determine the oral bioavailability, and to define the plasma concentrations associated with effective dosing regimens. Studies were conducted in conventional mice after IV, PO and SC doses of 50 mg/kg, and IP doses of 25 mg/kg, 50 mg/kg, and 100 mg/kg. In addition, plasma concentrations of 1-MT were determined in nude mice with implanted timed-release pellets designed to release the DL isomer for 7 days, and the D isomer for 14 days.

25 Peak plasma concentrations of D or L 1-MT were attained 2 hours after PO administration. The maximum concentration observed for the L isomer was approximately seven times greater than that for the D isomer. The AUC_{0-∞} for the L isomer was also substantially greater (9 fold) than that for the D isomer. Terminal disposition phase half-lives were similar for the two isomers, and also similar to those found after IV administration. The 30 oral bioavailability for D and L 1-MT were 64% and 105%, respectively.

The in vivo disposition of the D and L isomers of 1-MT are remarkably different considering their nearly identical chemical structures. Analysis of their plasma pharmacokinetics suggests that the basis for the difference is a rapid partitioning of the D isomer into tissues compartments other than the plasma, while the L isomer is more predominantly distributed in the 5 plasma. As a consequence, the AUC_{0-∞} observed for L 1-MT were from 5 to 9 times greater than those found for D 1-MT with equivalent routes of administration and doses. Given the comparatively lower plasma levels of the D isomer, while tempting to speculate regarding where the D isomer does reside, the experimental design of these studies did not encompass 10 examination of the tissue or cellular distribution of 1-MT. However, one possible explanation to account for the relatively greater apparent volume of distribution of the D isomer is a higher intracellular localization, raising the possibility of a unique cellular transport mechanism for D 1-MT not shared by L1-MT. Cellular uptake studies will be performed with the two isomers to 15 address this pivotal issue.

Both isomers were absorbed efficiently following IP, SC and PO administration, and the 20 plasma pharmacokinetics were comparable regardless of the route of administration, indicating that any of the routes studied are equally suitable for efficacy testing. The oral bioavailability of the D isomer (64%) was somewhat lower than that observed when it was given by the intraperitoneal (IP) or subcutaneous (SC) route, and also lower than the oral bioavailability of L 1-MT.

20

Pilot studies to address the tissue biodistribution of the D-isomer

Mice were given 1MT as the DL racemic mixture (10 mg/day, SQ implanted copolymer 25 pellets, 72 hour infusion), then tissues were harvested, extracted, and tissue levels of the respective D and L isomers determined by generation of diastereomeric isoindoyl derivatives with OPA and Boc-L-Cys (Hashimoto et al., *J Chromatography* 1992;582:41-48). Initial results showed at least equal, and possibly preferential, accumulation of the D isomer in liver, with equivalent distribution of D and L isomers into muscle and spleen. The results noted with spleen are particularly relevant because it is a lymphoid organ, and thus likely to be similar to tumor-draining lymph nodes. Initial results also showed lower levels of the D isomer in plasma and in 30 brain, suggesting that the D isomer may not cross the blood-brain barrier efficiently. Thus, the

lower plasma levels and shorter intravascular half-life do not necessarily imply lower levels in the relevant tissues.

Discussion

5 1-MT represents the lead compound in a new class of immunomodulatory agents, designed to block immunosuppression mediated by IDO. Many tumors, under the selection pressure of host immune surveillance, have evolved some means to exploit the tolerogenic activity of IDO, either directly, through expression of IDO by tumor cells, or by recruiting IDO-expressing APCs to induce systemic tolerance. Thus, 1MT will be indicated as an immunologic
10 10 adjuvant in combination with anti-tumor vaccines, including peptide, cell-lysate, or dendritic cell-based vaccines, and as an immunomodulatory agent in combination with chemotherapy or radiation. 1MT is a lead compound, representative of a new class of immunomodulatory drugs designed to inhibit the IDO pathway. It is one of a number of small-molecule, orally-bioavailable immunostimulatory agents.

15 The complete disclosure of all patents, patent applications, and publications, and electronically available material (including, for instance, nucleotide sequence submissions in, e.g., GenBank and RefSeq, and amino acid sequence submissions in, e.g., SwissProt, PIR, PRF, PDB, and translations from annotated coding regions in GenBank and RefSeq) cited herein are incorporated by reference. The foregoing detailed description and examples have been given for
20 20 clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claims.

 All headings are for the convenience of the reader and should not be used to limit the meaning of the text that follows the heading, unless so specified.

Sequence Listing Free Text

SEQ ID NO:1 peptide; human indoleamine-2,3-dioxygenase (IDO)
5 SEQ ID NO:2-7 Synthetic oligonucleotide primers